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(54) Title: METHOD AND KIT FOR DETERMINING SEVERE INFLAMMATORY REACTIONS TO MOSQUITO BITES

(57) Abstract

Mosquito extract consisting essentially of antigens solely to allergens in mosquito saliva. Isolated and purified recombinant mosquito saliva antigens for use in skin tests, immunoassays and immunotherapy for allergic reactions to mosquito bites. A method for skin testing and determining the undertaking of immunotherapy includes the steps of administrating the isolated and purified mosquito salivary antigens to a patient and then recognizing skin reaction thereto is a positive indication of needing therapy. A kit and an immunoassay are also provided for measurement of mosquito salivary allergen specific IgE and IgG wherein the assay includes the isolated and purified recombinant mosquito allergens as a substrate to which allergen specific IgE and IgG binds. Antibodies directed against the recombinant salivary allergens of mosquitoes are also disclosed.

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METHOD AND KIT FOR DETERMINING SEVERE INFLAMMATORY REACTIONS TO MOSQUITO BITES

CROSSREFERENCE TO RELATED APPLICATIONS

This application is a conversion of a Provisional Application filed November 13, 1997, Serial No. 60/065,402.

BACKGROUND OF THE INVENTION

Mosquito bites are a global problem not only because they facilitate transmission of potentially fatal diseases such as malaria and yellow fever, but also because they cause local skin reactions and, rarely, systemic reactions including urticaria, angioedema, and even anaphylactic shock [Frazier, 1973; McCormack *et al.*, 1995]. Skin reactions to mosquito bites are caused by the proteins in the mosquito saliva that enter the skin when mosquitoes take a blood meal [Hudson *et al.*, 1960]. Mosquito saliva proteins elicit both IgE-mediated immediate hypersensitivity and lymphocyte-mediated delayed hypersensitivity [Oka 1989; Peng *et al.*, 1996]. Mosquito salivary proteins are also involved with many aspects of the process of hematophagy which provide new perspectives for evaluating the transmission dynamics of pathogens [James, 1994].

Mosquito saliva contains a complex of proteins. Protein visualization techniques using gel electrophoresis and silver staining have revealed as many as 20 peptides in adult mosquito *Aedes aegypti* saliva [Racioppi and Spielman, 1987], which include α-amylase, anticoagulants, anti-TNF, apyrase, esterase, D7, α-glucosidase, and sialokinins [James, 1994].

Immunotherapy is successful in the treatment of mosquito allergy [Frazier, 1973; Gluck and Pacin, 1968; McCormack, 1995; Tager et al, 1969; Benaim-Pinto and Fassrainer, 1990], correlating with the observation that natural desensitization eventually occurs during long-term exposure to the bites [Mellanby, 1946; McKiel and West, 1961; Peng, et al, 1994, 1996]. This therapy is neither well studied nor widely used at least in part because commercially available mosquito extracts have never been standardized and contain many non-saliva proteins which may cause sub optimal efficacy and side effects.

Commercial extracts of mosquito saliva protein are available. Commercial mosquito extracts contain multiple proteins and antigens since they are whole body extracts (Example 1). Many of the antigens in these extracts are unrelated to the allergens in mosquito saliva. The antibodies exhibited in patients which are directed against these non-saliva antigens in the commercial extracts may have been induced by inhalation of insect particles or by being bitten by other insects whose antigens cross-reacted with mosquito body components leading to the formation of IgE and IgG antibodies against mosquito body antigens. Commercial mosquito extracts should be standardized given their extreme heterogeneity (Example 1) and purer mosquito extracts should be used in the diagnosis and immunotherapy of mosquito allergy. In addition to the poor diagnostic accuracy of skin testing, injection of crude extract may lead to the development of sensitization to some of its components [Hamilton, 1990]. It has further been demonstrated that the efficacy of immunotherapy is considerably improved by the use of purified antigens [Ewan, 1989].

Purification or isolation of each of the saliva proteins is required to improve studies of the diagnosis and immunotherapy of mosquito allergy, and studies of mechanisms of mosquito-transmitted diseases.

However, purification of each salivary protein from mosquito whole body extract is an extremely laborious task and may result in the potential loss of important allergens or their biologic activity during the necessary multiple purification procedures. Collection of mosquito saliva is time-consuming and labor-intensive and therefore is also impractical. Utilization of molecular techniques to clone and express mosquito salivary proteins would be useful in developing allergens for skin testing and immunotherapy. However, not all recombinant proteins can be used as allergens since when the proteins are expressed glycosylation patterns of the proteins are not always followed as well as other folding parameters so that it is not clear that recombinant proteins will elicit the correct immunological response including biological activity.

To date, two salivary gland cDNAs of *Aedes aegypti* which encode a 68 kDa protein (apyrase) and a 37 kDa one (D7) have been cloned [James *et al.*, 1991; Smartt *et al.*, 1995]. However, these proteins need to be characterized to determine if they are allergens, if they are shared by other mosquito species and whether they would be useful in a standardized extract for the use in skin tests and immunotherapy.

To facilitate studies of mosquito allergy additional recombinant proteins are needed that are not species-shared and which can be used as an allergen in a standardized extract for skin testing and immunotherapy (desensitization). In addition, recombinant proteins that are species-specific are also needed so that appropriate combinations of allergens can be made that are patient-specific.

SUMMARY OF THE INVENTION

According to the present invention, a recombinant mosquito salivary allergen for use in skin tests,

immunoassays and immunotherapy for allergic reactions to mosquito bites is provided. The recombinant allergen is produced by a cDNA encoding an IgE-binding protein or fragment thereof or analogue thereof which is found in mosquito saliva. In an embodiment the recombinant allergen is rAed a 1, a 68 kDa recombinant allergen, or rAed a 2, a 37 kDa recombinant allergen or rAed a 3, a 30 kDa recombinant allergen. The recombinant allergen may share common allergenicity among at least two species of mosquitos or may be species-specific.

The present invention also provides a method of skin testing and undertaking immunotherapy utilizing the recombinant salivary allergen and a kit for practicing the method of the invention. The recombinant allergens are selected to share common allergenicity among the mosquito species for which testing or treatment is required. In a preferred embodiment a combination of allergens with common specificities among species is used so as to effectively represent the mosquito species distribution of a wide geographic area for which testing and/or immunotherapy is needed.

The present invention also provides an immunoassay for measurement of mosquito salivary allergen-specific IgE and IgG using recombinant mosquito salivary allergen as the substrate to which the allergen-specific IgE and IgG binds. The present invention also provides a kit for the immunoassay including the appropriate recombinant allergen, antibody directed to the allergen and may also contain reference sera.

The present invention further provides antibodies directed against the recombinant salivary allergens, wherein the recombinant allergen is produced by a cDNA encoding an IgE-binding protein or fragment thereof or analogue thereof which is found in mosquito saliva. In an embodiment, the antibody is directed against rAed a-- 1, a 68 kDa recombinant allergen, or rAed a 2, a 37 kDa

recombinant allergen or rAed a 3, a 30 kDa recombinant allergen. The antibody may be polyclonal or monoclonal. The antibodies are used for immunoassays, purification and antigen standardization.

DESCRIPTION OF THE DRAWINGS

Other advantages of the present invention will be readily appreciated as the same becomes better understood by reference to the following detailed description when considered in connection with the accompanying drawings wherein:

Figure 1 shows the results of using nitrocellulose filters to characterize cDNA coding protein using specific mouse and human antibodies to the protein;

Figure 2 shows results for mean mosquito-specific IgE levels of three species and the subjects with or without immediate skin reaction to mosquito bites;

Figure 3 shows the IgE (left) and IgG (right) responses to the antigens of *Ae. vexans* in three subjects with severe skin reactions to the bites (strip number 1-3) and two subjects without skin reactions to the bites (strips number 4 and 5);

Figure 4 shows a comparison of IgE and IgG responses in three subjects;

Figure 5 shows the results of skin epicutaneous tests;

Figure 6 shows the results of testing of nine subjects with positive rAed a 1 reactions;

Figure 7 shows Western blot analysis showing inhibition of binding by addition of mosquito head and thorax extract in a dose-dependent manner;

Figure 8 shows Aed a 2 to be a species-shared allergen being present in saliva or salivary glad extracts;

Figure 9 shows individual IgE responses to allergens evaluated in twelve mosquito-allergic subjects living in Canada, USA and China as well as

five subjects who were not allergic to mosquito bites, immunoblotting being performed using two rabbit antibodies specific to recombinant mosquito salivary proteins; and

Figure 10 is an immunoblot using rabbit anti-Aed a 1 and Aed a 2 antibodies respectively.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

The present invention provides recombinant mosquito salivary allergens for use in skin tests, immunoassays and immunotherapy. The recombinant allergen is identified and produced by a cDNA encoding an IgE-binding protein or fragment thereof or analogue thereof which is found in mosquito saliva as is shown in the Examples herein.

The allergen is generally a protein or protein fragment or analogue thereof found in the saliva of mosquitos as described herein above. The allergen elicits an IgE response and may also elicit an IgG response. The fragment contains some or all of the epitopes on the whole allergen. It elicits an IgE response and may also elicit an IgG response. The recombinant allergen expresses the same epitopes, either sequence based or conformational, as the native allergen and shares the same antigenic functions as the native allergen. The antigenic functions essentially mean the possession of an epitope or antigenic site that is capable of cross-reacting with antibodies raised against a naturally occurring salivary protein. Further, the recombinant antigen must also have the same biologic activity, that is it must elicit an immune response *in vivo*.

The term "analogue" as used herein is defined as a variant (alternatively the terms alteration, amino acid sequence alteration, amino acid sequence variant can be used) with some differences in their amino acid sequences as compared to the native sequence of

salivary proteins, but functionally equivalent. Ordinarily, the analogue will be generally at least 70% homologous over any portion that is functionally relevant for eliciting an immune response. In more preferred embodiments the homology will be at least 80% and can approach 95% homology to the mosquito salivary protein sequence. The homology will extend over a region of at least nine contiguous amino acids. The amino acid sequence of an analog may differ from that of the native protein when at least one residue is deleted, inserted or substituted, but the protein retains its antigenic competence and biological activity *in vivo* in relation to eliciting an immune response. Differences in glycosylation can provide analogues.

Functionally equivalent refers to the biological property of the molecule and in this context means an *in vivo* eliciting of an immune response by a naturally occurring (native) salivary proteins. The antigenic functions essentially mean the possession of an epitope or antigenic site that is capable of cross-reacting with antibodies raised against a naturally occurring salivary protein and eliciting skin reactions as do the native salivary allergens. Biologically active (*in vivo activity)* means that the analogues share an antigenic function and elicit an immune response *in vivo*.

Any expression system may be used as is known in the art that will provide recombinant allergens or allergen fragments that express the eliciting epitopes and have activity *in vivo*. In a preferred embodiment the allergen is produced in a baculovirus expression vector system.

As exemplars, recombinant allergens rAed a 1, a 68 kDa recombinant allergen, and/or rAed a 2, a 37 kDa recombinant allergen and/or rAed a 3 (SEQ ID No:1), a 30 kDa are used. The allergens are named using the

rules as set forth in Larson and Lowenstein [1996].

The recombinant allergens can be selected such that they share common allergenicity among at least two species of mosquitos as shown in the Examples. Alternatively, the allergen is species specific.

The allergens used for skin testing are selected on the basis of the mosquito species distribution in the geographic area in which the patient to be tested is exposed or will be exposed. It is contemplated by the present invention that in one embodiment patients moving into new geographic areas or planning to vacation in a new geographic area will be skin tested to determine their sensitivity to the predominant mosquito species population of that area and can then be desensitized as described herein below.

As shown in the Examples herein below the allergens may be cross-reactive across several species or may be species-specific. The skin testing will utilize the recombinant allergens that effectively represent the mosquito species distribution for the geographic area in which the patient is exposed or will be exposed.

The concentration of each recombinant allergen used in skin testing will be determined using *in vivo* and/or *in vitro* standardization techniques. The standardization techniques have been described in detail previously [Ipsen et al. 1993] and are summarized herein below. Each recombinant allergen extract will be standardized against a standard reference to assure lot-to-lot consistency and relative potency of allergenic recombinant extracts.

In addition to, or alternatively, to the skin testing the response to the recombinant mosquito allergens can be determined by an immunoassay as is known in the art and described herein below. In general an ELISA for IgE is preferred but Western Blotting can also be used. The present invention also provides a kit including the appropriate recombinant

allergen, antibody directed to the allergen and may contain reference sera for the practice of the immunoassay.

The present invention also provides an allergy immunotherapy medicament which contains the recombinant mosquito allergens for use in immunotherapy (desensitization) as described herein below. The recombinant allergens are suspended in pharmaceutically acceptable carriers, diluents, adjuvants and/or vehicles as is known in the art of immunotherapy. These pharmaceutically acceptable carriers and the like are selected such that they do not react with the active ingredients of the invention and that the allergens retain their immunologically eliciting conformation and biological activity.

It should be noted that an alternative term for the immunotherapy medicament is "extract" which is generally used to indicate allergens (immunogens) that have been isolated or prepared from a native or natural source and not produced recombinantly. An example of an extract would be mosquito whole body preparations. However, the allergy immunotherapy medicament of the present invention can be referred to as a "recombinant extract".

The allergy immunotherapy medicament will contain at least one recombinant mosquito allergen. In general, the recombinant mosquito allergens are selected on the basis of the skin test results. The starting dose of the allergen can be determined by skin test endpoint-titration using a dose that is equal to 0.1 ml of the end-point dilution that initiates a skin reaction or other methods known in the art. The medicament will contain a combination of recombinant allergens that effectively represent the mosquito species distribution for the geographic area to which the patient is allergic and for which they need desensitization. --

The present invention provides a kit for skin

testing for allergy to mosquito bites and a kit for providing immunotherapy including recombinant mosquito salivary allergens. In general the kit includes recombinant allergens that are selected to share common allergenicity among the mosquito species common to the geographic area for which testing or immunotherapy is required. The kit may also include species-specific recombinant allergens for each mosquito species common to the geographic area for which testing and/or immunotherapy is required. In an embodiment, the kit includes recombinant allergens rAed a1, a 68 kDa recombinant allergen, rAed a2, a 37 kDa recombinant allergen and rAed a3, a 30 kDa recombinant allergen.

Since 1865, skin tests have been used to provide helpful confirmatory evidence for diagnosis of specific allergy. Skin tests include epicutaneous and intradermal tests. Detailed techniques have been previously described [Bousquet and Michel 1993]. Briefly, in the epicutaneous tests, drops of recombinant allergen extracts are placed approximately 2 cm apart on the volar surface of the forearm. The point of a disposable needle is passed through the drop, inserted into the epidermal surface, and then gently lifted without inducing bleeding. The immediate wheal and flare reactions are traced and recorded 20 minutes after the test, and delayed indurated papules are traced 24 hours later. In the intradermal tests, a volume of approximately 0.01 to 0.05 ml of the recombinant allergen is injected into the skin to produce a small superficial bleb approximately 2-3 mm in diameter. Results are traced and recorded in the same manner as for the epicutaneous tests.

Since 1911, immunotherapy injections with increasing amounts of the offending specific allergen(s), have been successfully used to relive allergic symptoms after subsequent exposure to the allergen(s). This therapy is still one of the major therapeutic methods for treatment of patients with

allergic diseases. The method is described in detail by TE van Metre and NFA Adkinson, Jr., [1993]. First, the recombinant mosquito allergens to which the subjects are allergic are determined by skin tests and/or measurement of specific IgE. These recombinant allergens are then combined and used for the immunotherapy. Subcutaneous injections of the allergens will be given weekly or twice a week in gradually increasing doses. A starting dose will contain 0.5 allergy units/ml or can be chosen by skin test end-point titration using a dose that is equal to 0.1 ml of the end-point dilution that initiate skin reaction. When the highest tolerated dose is reached, this dose is used to maintain allergen-specific immunity, that is, injected every 2 - 4 weeks for a period of time, as determined by clinical history and monitored immunologic tests.

Currently, native allergens (proteins) are used in immunotherapy. Recent studies have shown that immunotherapy with the genes (plasmid DNA) which encode for allergen inhibit specific IgE response, histamine release, and allergen-induced airway hyperresponsiveness [Hsu et al. 1996; Raz et al. 1996]. Therefore, immunotherapy with plasmid DNAs encoding or mosquito salivary allergens is contemplated by the present invention for immunotherapy for people who are highly reactive to mosquito bites.

Diagnosis of mosquito allergy can be also made by measurement of serum recombinant allergen-specific IgE and IgG antibodies using immunoassays. In general, ELISAs are the preferred immunoassays employed to assess the amount of IgE and IgG in a specimen. ELISA assays are well known to those skilled in the art. Both polyclonal and monoclonal antibodies can be used in the assays. Where appropriate, other immunoassays, such as radioimmunoassays (RIA) can be used as are known to those in the art. Available immunoassays are extensively described in the patent and scientific

literature. See, for example, United States patents 3,791,932; 3,839,153; 3,850,752; 3,850,578; 3,853,987; 3,867,517; 3,879,262; 3,901,654; 3,935,074; 3,984,533; 3,996,345; 4,034,074; 4,098,876; 4,879,219; 5,011,771 and 5,281,521.

Any expression system as is known in the art that will provide recombinant allergens or allergen fragments that express the eliciting epitopes and have activity *in vivo* can be used in the practice of the present invention. In a preferred embodiment the baculovirus insect cell expression system, which performs many of the post-translational modifications found in mammalian cells, is an excellent system for the production of large amounts of biologically active proteins (see generally O'Reilly et al, 1994. Baculovirus Expression Vectors: A Laboratory Manual. Oxford University Press). The efficiency of expression of baculovirus system differs from gene to gene by approximately 1000-fold. In particular this system provides for glycosylation such that proper carbohydrate expression is provided which plays a role in the immunological response and *in vivo* activity.

Vectors can be constructed containing the cDNA of the present invention, by those skilled in the art, and should contain all expression elements necessary to achieve the desired transcription of the sequences in the selected expression system. Other beneficial characteristics can also be contained within the vectors such as mechanisms for recovery of the nucleic acids in a different form. The vectors can also contain elements for use in either procaryotic or eucaryotic host systems. One of ordinary skill in the art will know which host systems are compatible with a particular vector.

The vectors can be introduced into cells or tissues by any one of a variety of known methods within the art (calcium phosphate transfection; electroporation; lipofection; protoplast fusion;

polybrene transfection). The host cell can be any eucaryotic and procaryotic cells, which can be transformed with the vector and which will support the production of the allergen with proper glycosylation and conformation.

pBlueBacHis vectors are designed for efficient expression and purification of recombinant proteins [Chen et al, 1993; Reddy et al, 1994; Rotrosen et al, 1993; O'Reilly et al, 1994; Matsuura et al., 1987; Rupp et al., 1995; Chalkley et al, 1994] and have been used by applicants. Since the variety of expression vector may also play a role in the expression levels of baculovirus system, expression vectors are selected for each recombinant allergen as is known in the art such that the yield of allergen is maximized.

Expressed recombinant allergens in the cell culture media will be purified according to the physio-biochemical characteristics of each recombinant allergen as is known in the art. For example, histidine tagging [Xu et al., 1996] for purification of recombinant proteins by immobilized metal affinity chromatography can be used for a number of proteins both in prokaryotic [Dudler et al., 1992.] and eukaryotic [Janssen et al., 1995; Reddy et al, 1994] expression systems. Other purification systems as are known in the art can be used including affinity chromatography utilizing the monoclonal antibodies to recombinant allergens of the present invention. Alternatively, a combination of ion exchange (DEAE Sephacel) and gel filtration (Sepgacryl S-100) chromatograph can be used. Whatever method of purification that is used will be selected such that the isolated and purified recombinant allergen will maintain its immunologic and biologic activity.

The present invention provides antibodies directed against the recombinant salivary allergens. These antibodies can be used in immunoassays and for purification and standardization of allergens. --

Applicants are producing mAb directed against recombinant allergens in BALB/c mice.

Antibodies (immunoglobulins) may be either monoclonal or polyclonal and are raised against the immunogen. Conveniently, the antibodies may be prepared against the immunogen or part of the immunogen for example a synthetic peptide based on the sequence, or prepared recombinantly by cloning techniques or the natural gene product and/or portions thereof may be isolated and used as the immunogen. Such immunogens can be used to produce antibodies by standard antibody production technology well known to those skilled in the art as described generally in Harlow and Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1988 and Borrebaeck, *Antibody Engineering - A Practical Guide*, W.H. Freeman and Co., 1992. Antibody fragments may also be prepared from the antibodies and include Fab, $F(ab')_2$, and Fv by methods known to those skilled in the art.

For producing polyclonal antibodies a host, such as a rabbit or goat, is immunized with the immunogen, generally with an adjuvant and, if necessary, coupled to a carrier; antibodies to the protein are collected from the sera. Further, the polyclonal antibody can be absorbed such that it is monospecific. That is, the sera can be absorbed against related immunogens so that no cross-reactive antibodies remain in the sera rendering it monospecific.

For producing monoclonal antibodies the technique involves hyperimmunization of an appropriate donor with the immunogen or immunogen fragment, generally from a mouse, and isolation of splenic antibody producing cells. These cells are fused to a cell having immortality, such as a myeloma cell, to provide a fused cell hybrid which has immortality and secretes the required antibody. The cells are then cultured, in bulk, and the monoclonal antibodies harvested from the

culture media for use.

For producing recombinant antibody (see generally Huston et al, 1991; Johnson and Bird, 1991; Mernaugh and Mernaugh, 1995), messenger RNAs from antibody producing B-lymphocytes of animals, or hybridoma are reverse-transcribed to obtain complimentary DNAs (cDNAs). Antibody cDNA, which can be full or partial length, is amplified and cloned into a phage or a plasmid. The cDNA can be a partial length of heavy and light chain cDNA, separated or connected by a linker. The antibody, or antibody fragment, is expressed using a suitable expression system to obtain recombinant antibody. Antibody cDNA can also be obtained by screening pertinent expression libraries.

The antibody or antibody fragment can be bound to a solid support substrate or conjugated with a detectable moiety or be both bound and conjugated as is well known in the art. (For a general discussion of conjugation of fluorescent or enzymatic moieties see Johnstone & Thorpe, *Immunochemistry in Practice*, Blackwell Scientific Publications, Oxford, 1982.) The binding of antibodies to a solid support substrate is also well known in the art. (see for a general discussion Harlow & Lane *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory Publications, New York, 1988 and Borrebaeck, *Antibody Engineering - A Practical Guide*, W.H. Freeman and Co., 1992) The detectable moieties contemplated with the present invention can include, but are not limited to, fluorescent, metallic, enzymatic and radioactive markers such as biotin, gold, ferritin, alkaline phosphatase, β-galactosidase, peroxidase, urease, fluorescein, rhodamine, tritium, ^{14}C and iodination.

Additionally, in the production of the antibody, a mimetope can be used as the antigenic source. That is a molecule having an epitope the same or similar to the antigenic determinant (epitopes) of interest may be

used as the source of the eliciting antigen.

The diagnosis of Skeeter Syndrome should be considered in any patient with cellulitis (a localized area of erythema edema, induration, pain and/or itch) at the site of a mosquito bite and negative cultures. Appropriate antibiotic treatment should be continued until a bacterial etiology for the inflammation has been ruled out with certainty; then, topical and/or oral glucocorticoids and an H_1-antagonist can be substituted.

While the long-term prognosis of Skeeter Syndrome is generally favorable, natural desensitization depends on the frequency and intensity of exposure and may take many years to develop.

The antigens in the kit of the present invention are found in the saliva of the mosquitoes *Aedes vexans* and *Aedes aegypti*, and are cross-reactive with other mosquito species. They have been characterized in our laboratory as follows:

Antigens:

There are more than 12 antigens in the saliva of *Aedes vexans*. Their molecular weight ranges between 17.5 and 75 kDa. There are more than eight antigens in the saliva of *Aedes aegypti*. Their molecular weight ranges between 18.5 and 68 kDa.

Antibodies:

Mosquito saliva antigens induce IgE, IgG, and especially IgG4 antibody responses which mediate the immediate and delayed type hypersensitivities in humans. These antibodies are found in patients with inflammatory reactions to mosquito bites (Skeeter Syndrome).

Relevance to Human Allergic Disease:

The *Aedes vexans* or *Aedes aegypti* salivary antigens are used in the ELISA immunoassays for measurement of mosquito saliva-specific IgE, IgG, and IgG4 antibodies, as known in the art.

These assays are applicable to patients presenting with rashes and other symptoms after mosquito bites, especially those with erythema, edema, and induration, pain or itch at the site(s) of mosquito bite(s), with or without fever. Such patients are at risk for severe localized inflammatory reactions and systemic reactions to mosquito bites.

We expect such patients to have antibodies in the range of 30 - 5,000 U/ml for specific IgE, 30 - 7,000 U/ml for specific IgG, and 50 - 33,000 U/ml for specific IgG4.

The above discussion provides a factual basis for the use of recombinant mosquito salivary proteins for use in skin testing and immunotherapy. The methods used with and the utility of the present invention can be shown by the following non-limiting examples and accompanying figures.

EXAMPLES

GENERAL METHODS:

Most of the general techniques described hereinbelow are widely practiced in the art, and most practitioners are familiar with the standard resource materials which describe specific conditions and procedures. However, for convenience, the following paragraphs may serve as a guideline.

General methods in molecular biology: Standard molecular biology techniques known in the art and not specifically described were generally followed as in Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Springs Harbor Laboratory, New York (1989), in Ausubel et al., *Current Protocols in Molecular Biology*, John Wiley and Sons, Baltimore, Maryland (1989) and in Perbal, *A Practical Guide to Molecular Cloning*, John Wiley & Sons, New York (1988).

General methods in immunology: Standard methods in

immunology known in the art and not specifically described were generally followed as in Stites et al.(eds), Basic and Clinical Immunology (8th Edition), Appleton & Lange, Norwalk, CT (1994) and Mishell and Shiigi (eds), Selected Methods in Cellular Immunology, W.H. Freeman and Co., New York (1980).

Laboratory-made mosquito Aedes aegypti preparations: An *Aedes aegypti* mosquito colony was obtained from the Department of Entomology, University of Manitoba, and maintained in applicants' laboratory. Female mosquitoes were used in the preparation of mosquito whole body, head and thorax, salivary gland, and saliva extracts. Saliva was collected from 3-15 day old adult mosquitoes by placing the proboscis of each mosquito into a capillary tube filled with water. Salivation was induced by applying 0.5 µl of 0.5% malathion in acetone to the thorax [Boorman, 1987]. One hour later, the contents of capillary tubes containing saliva were collected, pooled, and lyophilized. The saliva was reconstituted by dissolving the lyophilized proteins in 0.02 M phosphate buffered saline. In general, the protein concentration was 0.6 mg/ml for *Ae. vexans* extract, 0.4 mg/ml for *Cx. quinquefaciatus* extract, and 0.3 mg/ml for *Ae. aegypti* extract as measured by a Bio-Rad Protein Assay kit (Bio-Rad Labs, Richmond, CA).

Skin bite tests and blood samples: Skin bite tests were performed with one *Ae. vexans* mosquito and one *Ae. aegypti* mosquito on the volar aspect of each subject's forearm as previously described [Peng et al., 1996]. The wheal and flare circumferences were traced at 20 minutes and 24 hours after the bite, using a felt-tipped pen. All wheal and flare tracings were transferred to transparent paper. The area of the wheal, flare or induration was measured using an IBM-PC (IBM Instruments, Inc., Danbury, Conn.) digitizer and stereometric measurement software [Simons, et al, 1990].

A wheal of less than 0.3 cm^2 with no flare and no itch was considered to be a negative immediate reaction. An induration less than 0.3 cm^2 was considered to be a negative delayed reaction.

Preparation of mosquito salivary gland extract: Mosquitos (*Aedes vexans*), collected and identified in the Department of Entomology, University of Manitoba, were anaesthetized by chilling them at 4°C in a refrigerator. Salivary glands were dissected from female mosquitos in 0.02 M phosphate buffered saline (PBS), pH 7.2 under a binocular microscope and immediately transferred to 1 ml of PBS on ice. A total of 370 salivary glands were gathered in 1 ml of PBS, ultrasonicated for 30 seconds, and centrifuged at 8820 g for 15 minutes. The supernatant was collected, aliquoted, and stored at -70°C. The protein concentration of the antigen preparation was 0.6 mg/ml as determined by the Lowry method.

Measurement of human serum mosquito-specific IgE and IgG by ELISA: Mosquito-specific IgE and IgG in human sera were measured using indirect ELISAs. Standardization of ELISA results between assays and the estimation of relative amount of mosquito-specific IgE or IgG in each sample was accomplished by using reference IgE and IgG sera. These reference sera were obtained from one subject with a high value of mosquito-specific IgE and another subject with a high value of mosquito-specific IgG and were defined as 1,000 U/ml for mosquito-specific IgE and IgG antibodies, respectively. Polystyrene immunoplates (Nunc-Immuno Plate Maxisorp, Denmark) were coated with mosquito salivary gland extract (0.05 µg/well) diluted in 0.05 M carbonate buffer (pH 9.6) and incubated overnight at 4°C. After rinsing the plates three times with washing buffer (0.05% Tween 20 in 0.01 M PBS, pH 7.2) and blocking them with 2% bovine serum albumen (BSA) in washing buffer for one hour at room --

temperature, 0.1 ml of the test sample or of the reference serum diluted in ELISA buffer (0.2% BSA in washing buffer) was added to each well. The plates were incubated overnight at room temperature for IgE and 1.5 hours at 37°C for IgG. The wells were washed, and 0.1 ml of goat anti-human IgE (P.S. myeloma-affinity purified, a gift from Dr. N. F. Adkinson, Jr., The Johns Hopkins Allergy and Asthma Centre, USA) or goat anti-human IgG Fc fragment (Jackson ImmunoResearch Laboratories, Inc., PA, USA) diluted in ELISA buffer was added and incubated for 1.5 hours at 37°C. After washing, 0.1 ml of diluted enzyme-conjugated rabbit anti-goat IgG (Jackson ImmunoResearch Laboratories, Inc.) was added and incubated for 1.5 hours at 37°C. After a final wash, 0.1 ml of the enzyme substrate (1 mg/ml of *p*-nitrophenylphosphate in diethanolamine buffer, pH 9.8) was added and incubated overnight at 4°C. The reaction was stopped by addition of 0.1 ml of 1 N NaOH. Optical absorbance at 410 nm was read, using the THERMOmax microplate reader (Molecular Devices, CA, USA). The value of mosquito-specific antibodies was calculated by interpolation from the dilution curve of the reference serum.

Both IgE and IgG determinations were performed on two dilutions of serum, with each dilution duplicated. 50 samples were assayed twice for mosquito-specific IgE and IgG.

Inhibition tests: In order to examine the specificity of the assays, inhibition tests were conducted [Peng et al, 1995]. The mosquito salivary gland extract was 10-fold sequentially diluted in ELISA buffer. Each dilution of the extract was incubated with a diluted serum with high mosquito-specific IgE or high mosquito-specific IgG for two hours at 37°C and then followed by 4°C overnight. The final serum dilution was one in 20 for the inhibition of IgE and one in 200 for that of IgG. Incubation of the serum dilution with ELISA

buffer served as a control. Incubations of the diluted serum with 10-fold serial dilutions of grass pollen (Hollister-Stier, Miles Canada Inc., Ont.) also served as controls. Mosquito-specific IgE and IgG in these samples were then measured by ELISA as described above. these tests validated the ELISA immunoassays [Peng et al, 1995].

Statistical analysis: Analysis of data was performed using NCSS software. Unpaired *t* tests were used for between group comparisons. Linear regressions were used for the correlation of serum mosquito-specific IgE and IgG antibodies.

SDS-PAGE and silver stain: Proteins from mosquito extracts were separated by SDS-PAGE under reducing conditions in a discontinuous system using a Bio-Rad mini slab gel apparatus. One to two micrograms each of the laboratory-made mosquito whole body, head and thorax, salivary gland, and saliva extracts, were loaded onto different wells and electrophoresed in 10% acrylamide SDS-PAGE. Molecular weight protein standards (Bio-Rad) were used to determine the relative molecular weights of the electrophoresed components. Separated proteins were detected by silver staining (Bio-Rad Silver Stain kit).

SDS-PAGE and immunoblot analysis for IgE and IgG binding antigens: The proteins in the mosquito extracts were separated by SDS-PAGE in a discontinuous system according to Laemmli [1970] using a Bio-Rad slab gel apparatus. For each mosquito extract, fifteen micrograms of proteins prepared in a reducing buffer were loaded onto each well and separated by electrophoresis in 12% SDS-PAGE. These proteins were then electro-transferred onto nitrocellulose membranes. Free binding sites on the membranes were blocked by incubation with 3% bovine serum albumin (Sigma, St. Louis, MO) dissolved in 0.02 M PBS for two hours.

Immunoblot was completed by incubation of the

membranes with a pooled serum exhibiting high mosquito-specific IgE and IgG (1:10 dilution for IgE and 1:50 for IgG) over night. This was followed by incubation with monoclonal anti-human IgE (1:15,000) (ascites, clone No. 7.12, a gift from Dr. A. Saxon, Univ. of California) or monoclonal anti-human IgG (1:15,000) (PharMingen, CA) for 1.5 hours. After washing three times with PBS containing 0.05% (v/v) Tween 20, the membranes were incubated with horseradish peroxidase conjugated goat anti-mouse IgG (1:5,000 dilution for IgE and 1:10,000 for IgG) (Calbiochem Corporation, CA) for 1.5 hours. After washing, the membranes were incubated in ECL detecting reagents (Amersham Life Science, Buckinghamshire, England)) and then exposed to Kodak film (X-Omat, Kodak).

PBS and umbilical cord serum were used respectively to replace the pooled human serum as controls. Pre-stained SDS-PAGE standards (Bio-Rad, Richmond, CA) were used to determine the relative molecular weights of the electrophoresed components.

EXAMPLE 1

COMPARISON OF PROTEINS, IgE AND IgG BINDING ANTIGENS, AND SKIN REACTIVITY IN COMMERCIAL AND LABORATORY-MADE MOSQUITO EXTRACTS

In order to improve the precision of diagnosis of mosquito allergy, and to ensure the efficacy and safety of immunotherapy with mosquito extracts, applicants previously analyzed commercial mosquito extracts with respect to protein and antigenic composition, and biologic activity [Peng and Simons, 1995]. Seven commercially available mosquito whole body extracts supplied for skin testing and/or immunotherapy from six manufacturers were investigated. The commercial materials were compared with four mosquito extracts made in applicants' laboratory from mosquito whole body, head and thorax, salivary gland, and saliva. Epicutaneous tests, measurement of protein

concentration, sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) for protein components, and SDS-PAGE and immunoblotting for IgE and IgG binding antigens were performed with all commercial and laboratory-made extracts.

Commercial mosquito extracts: Seven commercial mosquito extracts available for epicutaneous tests and/or immunotherapy in mosquito allergy were purchased from six manufacturers. These extracts were made from *Culex* and *Aedes* genera. Information provided by the manufacturers about the extracts is listed in Table 1.

Epicutaneous tests and mosquito bite tests: Epicutaneous tests with the seven commercial mosquito extracts and the four laboratory mosquito preparations were performed on two subjects with severe skin reactions to *Aedes aegypti* bites and on two subjects with no skin reaction to the bites. Histamine phosphate (1 mg/ml), saline and 50% glycerin in saline were used as positive and negative controls, respectively. Mosquito bite tests were performed using female mosquitoes (*Aedes aegypti*) reared in the laboratory. Skin immediate wheal and flare reactions were measured 30 minutes after the tests and skin delayed papule reactions were measured 24 hours later using the largest and the orthogonal diameters of the wheal or the papule, respectively. The area of the wheal or papule was calculated after subtracting the area of the wheal or papule produced by the relevant negative control (if present).

Protein assay: Protein concentration of each commercial mosquito extract and laboratory mosquito preparations was determined by a Bio-Rad Protein Assay kit (Bio-Rad Labs, Richmond, CA).

SDS-PAGE and immunoblot analysis of IgE and IgG binding antigens: The pooled serum used in immunoblotting was obtained from six subjects exhibiting severe skin reactions to mosquito bites and high mosquito-specific

IgE and IgG as measured by ELISA. These subjects, including the two skin and bite test positive subjects, lived in Manitoba or Texas where the *Aedes* and/or *Culex* mosquito species are abundant.

In the comparison of commercial mosquito extracts, 10 µl of each commercial extract, 6 µl of *Aedes aegypti* saliva and 10 µl of *Culex quinquefaciatus* salivary gland extract were loaded onto different wells. Proteins separated by 12% SDS-PAGE were electrophoretically transferred onto nitrocellulose membranes. Free binding sites on the membranes were blocked by incubation with 3% bovine serum albumin in 0.05 M PBS Tween 20 for two hours. Immunoblotting was completed by incubation of the membranes with the pooled serum (1:10 dilution for IgE binding antigens, 1:50 for IgG binding antigens). After washing, this was followed by sequential incubations of the membranes with monoclonal anti-human IgE or monoclonal anti-human IgG (PharMingen, CA), and HRP-conjugated goat anti-mouse IgG (Calbiochem Corporation, CA). After washing, the membranes were finally incubated with ECL detecting reagents (Amersham Life Science, Buckinghamshire, England) and then exposed to the film (X-Omat, Kodak). Pre-stained SDS-PAGE standards (Bio-Rad, Richmond, CA) were used to determine the relative molecular weights of the electrophoresed components.

RESULTS

Epicutaneous tests and protein concentrations:

Immediate and delayed reactions were found at the sites of skin tests and mosquito bites in the two subjects allergic to mosquito bites. The immediate reaction was a pruritic wheal with a surrounding flare appearing within a few minutes, peaking at 30 minutes and then subsiding. The delayed papules were found several hours later, reaching a peak 24 hours after the epicutaneous and the bite tests. In contrast, there was little or no skin reaction to either the

epicutaneous sites or the bite site in the two control subjects.

Wide variations in both skin immediate and delayed reactions to the seven commercial mosquito materials were found in the two reactive subjects. The mean immediate wheal sizes ranged from 0.5 to 32 mm^2, and the mean delayed papule sizes ranged from 0 to 36 mm^2 (Table 2). Two aqueous extracts, C5 and C7, appeared to have no biological activity in humans. The protein concentration of the commercial extracts varied greatly, ranging between 0.09 and 4.85 mg/ml, but generally correlated with the size of skin reactions ($r = 0.72$, $p < 0.003$ for wheal; $r = 0.80$, $p < 0.005$ for papule). An exception to this was the C7 extract which had a protein concentration of 1.67 mg/ml, but elicited no skin response.

In order to evaluate the biological activity of each gram of protein, the ratio of skin reactions/protein concentration was calculated (Table 2). The skin reactivity per gram of protein varied from 0.3 to 14.9 in the seven commercial extracts. Among the four laboratory-made mosquito preparations, as the purity of the preparation increased, rank ordered from whole body, head and thorax, salivary gland to saliva, the skin reactivity per gram of protein increased significantly. Perhaps the skin reactivity per gram of protein in saliva (78.0) was less than that found in salivary gland (128.6) because the lyophilization required in the preparation of saliva extract reduced some biological activity of saliva.

SDS-PAGE and silver stain: In the four laboratory preparations, rank ordered from whole body, head and thorax, salivary gland to saliva extracts, the amount of salivary antigens significantly increased, while non-salivary proteins and antigens significantly decreased. There were 24 visible protein bands in the head and thorax extract, 16 in the salivary gland

extract, but only nine in the saliva extract. When commercial extracts containing the same amount of protein were used, the protein bands were obscure and the background was very dark.

SDS-PAGE and immunoblot analysis: The IgE and IgG binding antigens in the seven commercial mosquito extracts were measured. The antigens of the three commercial *Aedes* extracts were compared to the antigens in the *Aedes aegypti* saliva extract, while the antigens of the four commercial *Culex* extracts were compared to the antigens in the *Culex quinquefaciatus* salivary gland extract. *Culex quinquefasciatus* and *Culex pipiens* belong to the same genus and are sibling species. Because the *Culex* species does not salivate using the method applied to induce *Aedes* species to salivate, the salivary gland extract of *Culex quinquefaciatus* was used to compare antigens with the *Culex pipiens* whole body extracts. Among the seven commercial mosquito extracts both IgE and IgG binding antigens varied greatly both in the number of antigen bands and the amount of each antigen. Multiple antigens were found in the commercial extracts. Most of these antigens were not present in the saliva or salivary gland extract. Some extracts, C1, C2, C3, contained small amounts of saliva antigens. Two extracts, C5 and C7, contained no visible saliva or salivary gland antigens, although they did contain other proteins (0.09 and 1.67 mg/ml of proteins, respectively).

The IgE and IgG binding antigens in one commercial mosquito whole body extract and four laboratory-made mosquito preparations (whole body, head and thorax, salivary gland and saliva) were observed. Whole body, head, and thorax extracts contained multiple antigens which were not present in saliva, but they contained few of the antigens present in saliva, although more

micrograms of proteins were loaded for these extracts. As the purity of the preparation increased from whole body extract to saliva, the number of proteins and antigens decreased dramatically. This observation correlates with the results shown in Table 2, in which the salivary gland and saliva extracts have lower protein concentrations (0.14 and 0.25 mg/ml, respectively) and higher ratios of skin reactivity/protein (128.6 and 78.0, respectively) than those in the whole body (protein 1.2 mg/ml, ratio 9.2) and the head and thorax extracts (protein 0.62 mg/ml, ratio 17.7).

DISCUSSION: Using both *in vitro* and *in vivo* tests, considerable variation was found in the commercial mosquito extracts. Indeed, two (C5 and C7) of three aqueous extracts (C4,C5,C7) elicited neither immediate nor delayed skin reactions and presented no visible antigen band in immunoblot. All four extracts preserved by 50% glycerin exhibited skin reactivity and visible protein bands suggesting that the extracts preserved in 50% glycerin are more potent and stable.

Commercial mosquito extracts contained multiple proteins and antigens, many of which are unrelated to the antigens in mosquito saliva. The antibodies observed in the human subjects directed against these non-saliva antigens in the commercial extracts may have been induced by inhalation of insect particles or by being bitten by other insects whose antigens cross-reacted with mosquito body components leading to the formation of IgE and IgG antibodies against mosquito body antigens.

These results suggested that commercial mosquito extracts should be standardized and that purer mosquito extracts should be used in the diagnosis and immunotherapy of mosquito allergy. In addition to having poor diagnostic accuracy in skin testing, injection of crude extract may lead to the development of sensitization to some of its components (Hamilton,

1990].

Standardization of the extracts is required because the process of collecting or extracting mosquito saliva is extremely tedious, it is currently impractical to use mosquito saliva or salivary gland extracts in diagnosis or immunotherapy. Therefore if commercially available mosquito extracts are to be used they must be standardized to increase the quantity of the active materials they contain. Standardization of antigen preparation is usually achieved by comparison of overall activity or major components of the extract with those of a reference preparation. An in-house reference mosquito extract and a pooled serum for each mosquito genus are used to evaluate the relative biological activity and the lot-to-lot variation of different batches of mosquito extracts including species specific and non-specific components. However, this procedure is not efficient and better means of standardized extracts are required. The present invention provides the use of recombinant salivary allergens to be used as a "recombinant extract" to simplify the need for standardization and to provide greater safety in immunotherapy.

EXAMPLE 2

CROSS-REACTIVITY OF SKIN AND SERUM SPECIFIC IgE RESPONSES AND ALLERGEN ANALYSIS FOR THREE MOSQUITO SPECIES WITH WORLD-WIDE DISTRIBUTION

In order to improve diagnosis and immunotherapy of mosquito allergy, purified or recombinant mosquito saliva antigens should be used as shown in Example 1. In order to do so, it should be determined if there are any cross-reactive skin and IgE responses and species-shared antigens among various mosquito species, especially those with world-wide distribution.

Ae. vexans, *Ae. aegypti*, and *Cx. quinquefasciatus*

are the three most important mosquito species distributed globally. *Ae. aegypti* (see generally Christophers, 1960. *Aedes aegypti* (L.) the yellow fever mosquito: its life history, Bionomics and Structure. London: Cambridge University Press) and *Cx. quinquefasciatus* (see generally Knight and Stone, 1977. A Catalog of the Mosquitoes of the World. 2nd ed. Washington: Ent Soc Am (Thomas Say Found.)) are found throughout the tropical regions of the world within 20°C isotherms, and *Ae. vexans* is found in North America, Eurasia, Asia, and Africa [Wood et al, 1979]. Comparison of the human immunological response to the three species has never been made. Because of climate, *Ae. aegypti* and *Cx. quinquefasciatus* are not present in Canada, while *Ae. vexans* is the major pest in Canada representing up to 80% of the local mosquito population. In order to determine whether the three mosquito species have cross-reactive immunological responses and species-shared antigens, skin bite tests were performed and serum mosquito specific IgE was evaluated for the three mosquito species in 41 Manitobans who had been exposed to *Ae. vexans* bites, but not to *Ae. aegypti* and *Cx. quinquefasciatus* bites. Species-shared allergens were also analyzed by immunoblotting, using the sera from mosquito-allergic subjects and an antibody to a recombinant *Ae. aegypti* salivary protein.

MATERIALS AND METHODS

Subjects: This project was approved by The University of Manitoba Faculty Committee on the Use of Human Subjects in Research, and the participants gave written, informed consent before study entry. Forty-one healthy subjects (21 males and 20 females), age 19 to 57 years, with skin reactions to mosquito bites ranging from none to strongly positive were recruited during the summer of 1993. All the subjects had lived

in Canada more than two years, and 71% had lived in Canada (mostly in Manitoba) since birth. Antihistamines and other medications which might suppress the skin bite test were withheld for an appropriate length of time before the study.

Mosquitoes, mosquito saliva and salivary gland extracts: Female *Ae. vexans* mosquitoes were collected in local fields and identified by scientists in the Department of Entomology, University of Manitoba. The *Ae. aegypti* colony was obtained from the same Department and maintained in our laboratory. The *Cx. quinquefaciatus* colony was imported from Dr. Robert J. Novak's laboratory, University of Illinois, Champaign, IL, and maintained in our laboratory. Four to twelve day old adult *Ae. aegypti* mosquitos were used for the bite tests and for saliva collection. Salivary glands were dissected from four to twelve day old adult *Cx. quinquefaciatus* mosquitoes. Mosquito saliva or salivary gland extracts were prepared for use in the ELISA and immunoblot as described herein above.

ELISA: Serum mosquito-specific IgE to *Ae. vexans*, *Ae. aegypti*, and *Cx. quinquefaciatus* were measured by an indirect ELISA as described herein above. Optimal conditions for dilutions of the 3 mosquito extracts, serum samples, goat anti-human IgE, and conjugated rabbit anti-goat IgG were chosen by checkerboard titration. Standardization of ELISA results between assays and estimation of the relative amount of mosquito-specific IgE in each sample was accomplished by using reference sera as described. The reference serum used to measure *Ae. aegypti*-IgE and *Cx. quinquefaciatus*-IgE was obtained from a subject with systemic reactions to mosquito bites (kindly provided by Dr. R.J. Engler, Walter Reed Army Medical Centre, Washington, D.C.). Another reference serum used to measure *Ae. vexans*-IgE came from a Manitoban with --

severe skin reactions to mosquito bites (immediate wheal 1.5 cm^2 and flare 11.6 cm^2). Both reference sera were defined as 1000 U/ml for mosquito-specific IgE. Microplates coated with mosquito saliva or salivary gland extract (0.02 - 0.05 µg/well) were sequentially incubated with serum samples (1:20) or reference serum (2-fold dilutions from 1:20 to 1:10,240), 1:1,000 goat anti-human IgE (P.S. myeloma-affinity purified, a gift from Dr. N.F. Adkinson, Jr., The Johns Hopkins Allergy and Asthma Centre), 1:1,000 alkaline phosphatase-conjugated rabbit anti-goat IgG (Jackson ImmunoResearch Laboratories, West Grove, PA). The values of mosquito-specific IgE in the tested samples were calculated by interpolation from the dilution curve of the reference serum. The sensitivity of the ELISAs was 0.8 U/ml.

ELISA inhibition tests: In order to study the cross-reactivity among the three species, ELISA inhibition tests were performed. A serum with high mosquito-specific IgE (final dilution 1:20) was incubated with serially diluted *Ae. vexans*, *Ae aegypti*, *Cx. quinquefaciatus* extracts with final dilutions of 1:4 and 1:20 at room temperature for one hour and then 4°C overnight. Incubation of the serum with ELISA buffer served as a positive control. These incubated materials were then measured for *Ae. aegypti*-IgE using ELISA.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis: Among the 41 subjects studied, sera from six subjects with large immediate skin wheal responses (> 1.0 cm^2) in the bite tests and high Ae. vexans-specific IgE levels (> 1,000 U/ml) were pooled and used for immunoblotting. SDS-PAGE and immunoblotting were performed as described herein above and in Peng et al., 1996. In the analysis of IgE and IgG antigens, two µg of the proteins from each mosquito extract were loaded and electrophoresed in 12% acrylamide SDS-PAGE under reducing conditions. Proteins separated by SDS-PAGE

were electrophoretically transferred onto nitrocellulose membranes. Free binding sites on the membranes were blocked by incubation for two hours with 3% bovine serum albumin in 0.02 M phosphate buffered saline, pH 7.4, containing 0.05% (v/v) Tween 20 (PBS-T). After washing three times with PBS-T, the membranes were incubated overnight with the pooled serum (1:10 dilution for IgE and 1:50 for IgG) and washed again. Incubation of the membranes with PBS-T served as a negative control. This was followed by sequential incubations with 1:15,000 monoclonal anti-human IgE (clone 7.12, from Dr. A. Saxon's laboratory, University of California) or 1:15,000 monoclonal anti-human IgG (PharMingen, San Diego, CA), and then HRP-conjugated goat anti-mouse IgG (1:5,000 for IgE, 1:10,000 for IgG) (Calbiochem Corporation, La Jolla, CA). After washing, the membranes were finally incubated with ECL detecting reagents (Amersham Life Science, Buckinghamshire, England) and then exposed to the film (X-Omat, Kodak). Pre-stained SDS-PAGE standards (Bio-Rad, Richmond, CA) were used to determine the relative molecular weights of the electrophoresed components.

The same method, with some modifications, was used in the study of a recombinant *Ae. aegypti* saliva protein, which was kindly provided by Dr. A. James at the University of California, Irvine, CA (James et al, 1991]. One µl of the baculovirus medium containing the recombinant protein and 2 µg of each mosquito extract were loaded onto different wells and electrophoresed. Medium from cells infected with wild-type baculovirus was used as a control. The membranes containing separated proteins were then incubated with rabbit antibody to the recombinant protein (1:5,000) (from Dr. A. James) followed by incubation with HRP-conjugated goat anti-rabbit IgG (1:5,000). The remaining steps were the same as the immunoblot using human serum.

Statistical analysis: Analysis of the data was performed using the "Number Crunch Statistical System" software. Unpaired *t* tests were used between group comparisons. Linear regressions were used for the analysis of correlations among skin reactions and serum mosquito-specific IgE levels.

RESULTS

Skin bite reactions: No systemic reactions were noted after the bite tests. Immediate and delayed skin reactions to mosquito bites were observed. The immediate reaction consisted of a pruritic wheal with a surrounding flare or erythema appearing within several minutes, reaching a peak at 20 - 30 minutes and then subsiding. The delayed reaction consisted of an indurated papule which appeared several hours later, and was peaked 24 - 36 hours and diminished over several days after the bite. In five subjects with severe delayed reactions, vesicles were found in the centres of the indurated areas, precisely in the area where the immediate wheal had been.

Twenty nine of the 41 subjects had positive immediate skin reactions to *Ae. vexans* bites. Twenty two of the 29 also reacted to *Ae. aegypti* bites. Seven subjects reacted to *Ae. vexans* bites only. The size of the immediate reactions ranged from 0 to 3.1 cm^2 for the wheal and 0 to 23.0 cm^2 for the flare, and 0 to 29.5 cm^2 for delayed reactions.

In the immediate reaction, wheals correlated significantly with flares in both species ($r = 0.72$, $p < 0.00$ for *Ae. vexans*; $r = 0.88$, $p < 0.00$ for *Ae. aegypti*). Also, immediate wheal and flare reactions showed significant correlation with the delayed reactions in each species as well (r's between 0.43 and 0.61, p's < 0.00). More interestingly, significant correlations of skin reactions were found between the two species, especially between the immediate wheal

sizes of the two species (r = 0.84, p < 0.00) and the delayed induration reactions of the two species (r = 0.77, p < 0.00).

Mosquito-specific IgE levels: The geometric mean *Ae. vexans*-IgE, *Ae. aegypti*-IgE, and *Cx. quinquefaciatus*-IgE were all significantly higher in the subjects with immediate skin reactions to *Ae. vexans* bites than in those with no immediate skin reaction to the bites (p's < 0.05). Similar results were found for mean mosquito-specific IgE levels of the three species in the subjects with or without immediate skin reactions to *Ae. aegypti* bites (Figure 2 bottom). Significant correlations were also found among the IgE levels of the three species (r's between 0.35 to 0.60, p's < 0.03).

Correlations among skin reactions and the IgE levels: There was inter-correlations among skin reactions and IgE levels of the three species. As expected, *Ae. vexans*-IgE values correlated significantly with skin reactions to *Ae. vexans* bites, and *Ae. aegypti*-IgE values correlated significantly with skin reactions to *Ae. aegypti* bites. *Ae. vexans*-IgE also significantly correlated with skin reactions to *Ae. aegypti* bites. The same correlation was found between *Ae. aegypti*-IgE and the delayed skin reactions to *Ae. vexans*, and between *Cx. quinquefaciatus*-IgE and both immediate and delayed reactions to *Ae. vexans* bites.

ELISA inhibition tests: The cross-reactive immunological responses among the three species were further confirmed by ELISA inhibition tests. *Ae. aegypti*-specific IgE reactions could be inhibited by incubation of the serum with all three extracts in a dose-dependent manner, confirming the existence of species-shared antigens among the extracts of the three species.

SDS-PAGE and immunoblot analysis: Immunoblot analysis further revealed the existence of species-shared antigens. Using the pooled serum from Manitobans allergic to mosquitos, the IgE and IgG antibodies not only bound to *Ae. vexans* antigens, but also to the antigens of the two species which are not found in Manitoba. There were no antigen bands found in the PBS control strips. Further, two bands in *Ae. aegypti* and *Cx. quinquefaciatus* extracts and one band in *Ae. vexans* extract were recognized by the rabbit antibody to the recombinant protein, suggesting that the 37 Kda protein of *Ae. aegypti* is also present in the salivary glands of *Cx. quinquefaciatus* and *Ae. vexans*.

Cross-reactive IgE responses have not been previously reported in mosquito allergy. Unlike other studies of cross-reactivity to insect bites or stings, we selected a specific location where *Ae. aegypti* and *Cx. quinquefasciatus* are not present, while *Ae. vexans* is the major pest representing up to 80% of the indigenous mosquito population. This allowed us to exclude the sensitization caused by the other two mosquito species.

The immunologic basis for the reactive skin and IgE responses among different mosquito species is the existence of species-shared antigens which are based on their identical protein sequences. Salivary secretions have been demonstrated to be directly responsible for skin reactions to mosquito bites. In the present Example, immunoblot analysis using saliva or salivary gland extracts, a number of species-shared antigens and several *Ae. vexans*-specific antigens were found.

Using the antibody to the recombinant protein, it was determined that the 37 kDa *Ae. aegypti* saliva protein is present in *Ae. vexans* and *Cx. quinquefaciatus* salivary gland extracts, further confirming the existence of species-shared antigens in

the three species. These species-shared antigens may well explain the cross-reactivity of skin reactions and IgE responses among different mosquito species.

EXAMPLE 3

IMMUNOBLOT ANALYSIS OF IgE AND IgG BINDING ANTIGENS IN EXTRACTS OF MOSQUITOES *AEDES VEXANS, CULEX TARSALIS* AND *CULISETA INORNATA*

Reactions to mosquito bites are generally caused by immunologic mechanisms, with both type I (IgE-mediated) and type IV (cell-mediated) hypersensitivities being involved [Oka K, 1989; Peng et al, 1996; Reunala et al, 1994a; 1994b]. Serum mosquito-specific IgE has been demonstrated to correlate with cutaneous mosquito bite reactions [Oka K, 1989; Peng et al, 1996]. Mosquito-specific IgG has also been found to correlate with skin mosquito bite reactions, suggesting that IgG may also be involved in the development of mosquito allergy [Peng et al, 1996].

Mosquito antigens have been identified by immunoblot analysis. A number of mosquito antigens with molecular masses ranging from 14 to 126 kDa have been reported in various mosquito species [Penneys et al, 1989; Shen et al., 1989; Wu and Lan, 1989; Brummer-Korvenkontio, 1990, 1994]. In this Example antigens are analyzed using immunoblot techniques on three mosquito species not previously examined; *Aedes (Ae.) vexans*, a globally distributed species (and the major pest species in Manitoba), and two North American species *Culex (Cx.) tarsalis* and *Culiseta (Cs.) inornata*.

Subjects: This study was approved by The University of Manitoba Faculty Committee on the Use of Human Subjects in Research, and the subjects gave written, informed consent before participation. Forty-two subjects with a history of local reactions to mosquito bites were recruited during the summer of 1993. Skin mosquito *Ae.* --

vexans bite tests were performed and serum mosquito (*Ae. vexans*) salivary gland-specific IgE and IgG antibodies (mosquito-specific IgE and IgG) were measured by ELISA in 42 subjects. Three subjects with severe skin reactions in the bite tests who also exhibited high mosquito-specific IgE and IgG levels, and two subjects with no skin reaction to the bites and with low mosquito-specific antibody levels, were selected for this study.

Skin reactions in the bite tests and antibody levels in the five subjects studied are listed in Table 3. The reactive subjects had very strong skin immediate reactions which consisted of a pruritic wheal (≥ 1 cm) with surrounding flare appearing within several minutes, reaching a peak at 30 minutes and then subsiding. The immediate reaction was followed by a local pruritic and indurated papule and erythema (≥ 7 cm) which appeared several hours later, reaching a peak 24 hours after the bite. The papule usually lasted several days.

A pooled serum from reactive subjects was used in the analysis of antigens in the three mosquito species.

Mosquito head and thorax extracts: Extracts prepared from mosquito heads and thoraxes were used in the study. Female mosquitoes of the three species (*Ae. vexans*, *Cx. tarsalis*, *Cs. inornata*) were collected and identified by the Insect Control Branch, Parks and Recreation Department, City of Winnipeg, and then stored at -70°C. After the abdomens, wings and legs were removed, the heads and thoraxes were placed in cold 0.02 M phosphate buffered saline (PBS), pH 7.2, homogenized on ice for 1 minute using a PCU 11 homogenizer (Kinematica, Switzerland), centrifuged at 8820 *g* for 30 minutes, filtered through a 0.45 µm Amicon filter, and then stored at -70°C. The protein concentrations of the antigen preparations were 1.48

mg/ml for *Ae. vexans*, 6.13 mg/ml for *Cx. tarsalis* and 5.34 mg/ml for *Cs. inornata*, as measured by the Protein Assay Dye Reagent kit (Bio-Rad Laboratories, Richmond, CA).

SDS-PAGE and immunoblot analysis for IgE and IgG binding antigens: As described herein above.

RESULTS

Inhibition test: To examine the specificity of the immunoblot analysis, an antigen inhibition test was performed. Prior to immunoblotting, the pooled serum with high mosquito-specific IgE and IgG was incubated with *Ae. vexans* head and thorax extract with a final dilution of 1:2, 1:20 or 1:200 at 4°C overnight. Incubation of the serum with PBS served as a positive control. SDS-PAGE and immunoblotting for IgE and IgG binding antigens was then performed with these pre-incubated serum samples. After incubation with mosquito extract, both IgE and IgG binding bands were significantly reduced compared to the positive control. This inhibition exhibited dose-dependency demonstrates that the immunoblot analysis of IgE and IgG binding antigens is specific to mosquito antigens.

Antigens in the three mosquito species: IgE and IgG antibodies bound to various mosquito antigens in the three species. Twelve antigens in *Ae. vexans*, 16 antigens in *Cx. tarsalis*, and 14 antigens in *Cs. inornata*, with molecular masses ranging from 18.5 to 160 kDa, were found by immunoblot analysis (Table 4). Most of the antigens bound to both IgE and IgG, and were shared by species. Nine antigens (24, 32.5, 40, 46, 50, 62, 65, 110, 160 kDa) were shared by three species, especially the 40 kDa antigen. Six antigens (28, 37, 43, 56, 70, 85 kDa) were shared by two species. Only three antigens were species-unique: the 18.5 kDa in *Ae. vexans*, the 25 kDa in *Cs. tarsalis*, and the 30 kDa in *Cs. inornata*. --

IgE and IgG antibody responses to Ae. vexans antigens in 5 subjects with or without skin reactions to mosquito bites: Figure 3 shows the IgE (left) and IgG (right) responses to the antigens of *Ae. vexans* in three subjects with severe skin reactions to the bites (strip #1-3) and two subjects without skin reactions to the bites (strips #4 and #5). In the five subjects, the patterns (spectra) of IgE and IgG responses to the antigens were similar, but the magnitudes of the antibody responses varied suggestively. Significantly strong IgE and IgG responses to the antigens, especially to the 32.5, 40, 43, and 50 kDa antigens, were found in the 3 reactive subjects (#1-3), while only very faint IgE antibody responses were observed in the 2 non-reactive subjects (#4,5), and very faint IgG responses to the antigens in 1 non-reactive subject (#5).

The patterns of IgE and IgG responses to the antigens varied slightly among the three reactive subjects. Some individuals reacted to certain antigens while others reacted to different antigens, eg. only subject #3's IgE bound to the 43 kDa antigen, and the IgE of subject #2 was the only one to bind to the 80 kDa antigens (Figure 3, indicated by arrows).

No antibody binding was found when the antigen containing membranes were incubated with either the cord serum (#6) or PBS (#7).

Comparison of IgE and IgG responses in three subjects: In order to define any differences between IgE and IgG responses to mosquito antigens in the same individual, the immunoblot results displaying IgE and IgG binding antigens were placed side by side for the three subjects with high mosquito-specific IgE and IgG antibodies (Figure 4). Although the patterns of antibody responses were slightly different among the three subjects, all the antigens induced both IgE and IgG responses. --

In summary, extracts of one globally distributed

mosquito species (*Aedes vexans*) and two North American species (*Culex tarsalis* and *Culiseta inornata*) were prepared from heads and thoraxes. Proteins of the three extracts were separated by 12% SDS-PAGE and transferred to nitrocellulose membranes for immunoblotting. Immunoblotting was completed by sequential incubations of the membranes with a pooled human serum from subjects allergic to mosquito bites, monoclonal antibodies to human IgE or IgG, and goat anti-mouse IgG conjugate. Twelve to sixteen antigens with molecular masses ranging from 18.5 to 160 kDa were found in each extract. Nine antigens were shared by three species and six were shared by two species. Only three were species-unique. Most antigens bound to both IgE and IgG antibodies.

IgE and IgG antibodies against *Aedes vexans* were studied by immunoblotting using individual serum from subjects with or without skin reactions to *Aedes vexans* bites. All three subjects with severe skin reactions had strong IgE and IgG antibodies to 32.5, 40, and 50 kDa proteins. The patterns and magnitudes of IgE and IgG antibodies to the antigens varied among individuals. Very faint IgE antibodies to these antigens were found in the 2 subjects with no skin reactions, suggesting that IgE plays a role in the development of mosquito allergy.

EXAMPLE 4

ISOLATION OF A cDNA ENCODING A 30 kDa IgE-BINDING PROTEIN OF MOSQUITO *AEDES AEGYPTI* SALIVA

Isolation of cDNA clone: Using mouse antiserum against *Aedes aegypti* saliva, produced as previously described [Yang *et al.*, 1997], with alkaline phosphatase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Lab. Inc, West Grave, PA, USA) a salivary gland cDNA library of mosquito *Aedes aegypti* (kindly provided by

Dr. A. James, University of California, Irvine) was screened according to the lambda library protocol (Clontech lab, CA, USA). Thirty-nine clones were isolated from about 120,000 plaques. These clones were grouped into A, B and C, according to the color strength of their reactions to the mouse antiserum on the filters. Four clones from each group were chosen to prepare fusion proteins using the method previously described [Huynh *et al.*, 1985].

Twelve samples of the fusion protein were separated by SDS-PAGE and immunoblotted by mouse anti-saliva serum. A protein band with a molecular weight ranging from 125 to 175 kDa was found in each sample (data not shown). As the ß-galactosidase part of the fusion protein is 114 kDa, the cDNA coding part ranged from 10 to 60 kDa. Three clones with different size of cDNA inserts were subcloned into pBluescript II SK vector (Stratagene, La Jolla, CA, USA) and sequenced by the Sanger method [1977] using a US Biochemicals sequencing kit (Cleveland, Ohio, USA). One clone (AA22) with a 0.75 kb of insert has a complete 3' terminus, which is consistent with known eukaryotic genes, a consensus polyadenylation sequence, AATAAA, and polyadenosines (Table 4; SEQ ID Nos:1). An open reading frame for 217 amino acids (estimated to be 25 kDa) was observed in frame with the ß-galactosidase protein, but this clone lacked an initiation codon in the 5' terminal sequence.

PCR was designed to clone the 5' terminal fragment from the cDNA library using the lambda gt11 forward primer (5' GACTCCTGGAGCCCG 3', Clontech; SEQ ID No:2) and a synthesized 3' primer (3'ATATCTGTCCACCAACG 5'; SEQ ID No:3) complementary to a sequence of the 3' terminal fragment. PCR was performed in 100 µl of the sample containing 0.5 µg of library DNA, 10 µl of 10× buffer, 2 µl of 25 mM dNTP's, 2 µl of 100 ng/µl each primer, 2 U of Taq polymerase supplied by the PCR kit

(Boehringer Mannheim Canada, Quebec). The reaction was subjected to 25 cycles of amplification consisting of 1 minute at 94°C, one minute at 55°C and one minute at 72°C, with a final 72°C extension for seven minutes. The PCR product was cloned into the TA vector (Invitrogen, San Diego, CA, USA).

Three PCR clones were obtained and sequenced. The sequences of the three clones were found to be identical and overlapped AA22 cDNA. In the search for an initiation codon in the same open reading frame as AA22 cDNA, an ATG was found, which was characterized with an adenosine at the crucial -3 position of the Kozak consensus sequence, A/GXXXATG, for initiation of translation by eukaryotic ribosomes [Kozak, 1987]. The sequence flanking the putative translational start sites, GAAAATG, is very similar to the consensus sequence, C/AAAA/CATG, for initiation of *Drosophila*, an insect gene (Cavener, 1987]. The full-length cDNA is 0.85 kb, coding for a protein of 253 amino acid residues, approximately 30 kDa.

Characterization of the cDNA coding protein: Specific mouse and human antibodies to the cDNA coding protein were prepared using the methods previously reported [Lyon *et al.*, 1986; Caraballo *et al.*, 1996]. Nitrocellulose filters were saturated with the fusion protein by overlaying the filters onto the lawn of *E. coli* Y1090 which had been previously infected by lambda AA22, and then incubated with mouse anti-*Aedes aegypti* saliva serum or a pooled mosquito-allergic human serum. Fusion protein-selected mouse or human antibodies were eluted from the filters by incubation of the filters with glycine buffer. These antibodies were used to probe the saliva proteins of *Aedes aegypti*. As shown in Figure 1, many bands were revealed in the immunoblotting with mouse anti-saliva serum (lane 1). When immunoblotting was completed with the fusion protein-selected mouse antibodies, only a 30 kDa

protein was specifically revealed (Figure 1, lane 2). The fusion protein-selected mouse antibodies recognize the 30 kDa saliva protein, indicating that this cDNA clone encodes the 30 kDa saliva protein.

As shown in the Examples, the 30 kDa saliva protein of mosquito *Aedes aegypti* elicited an IgE response in 42% of mosquito-allergic subjects and in none of the subjects without skin reactions to the bites. In Figure 2, the fusion protein-selected human IgE strongly bound to the 30 kDa native protein (lane 2), suggesting that the cDNA isolated codes the 30 kDa salivary allergen which induces a specific IgE response in mosquito-allergic humans. This IgE-binding protein is the third salivary allergen of *Aedes aegypti* whose cDNA has been cloned and sequenced.

From the searches of the DNA and protein databases to determine the identity of the cloned cDNA using BLAST network service [Altschul *et al.*, 1990], it is apparent that this putative protein represents a novel protein. Although the BLASTN results based on the nucleotide sequence indicate a high degree of similarity to a number of known sequences, these similarities most likely result from a number of repetitive codons in the sequence (data not shown). This interpretation is confirmed by the BLASTP search based on the conceptual translation product of the cDNA, which does not indicate any similarity to a known protein. The protein is rich in glutamic acid residues (16.5% of amino acid residues), and has a hydrophobic amino terminal region characteristic of a secretory signal peptide [Hopp *et al.*, 1981; Kyte *et al.*, 1982].

EXAMPLE 5

EXPRESSION, PURIFICATION, IMMUNOLOGICAL CHARACTERIZATION, AND CLINICAL USE OF rAed a 1, A 68 kDa RECOMBINANT SALIVARY ALLERGEN OF MOSQUITO *AEDES AEGYPTI*

Mosquito salivary proteins cause allergic reactions in humans. Aed a 1, a 68 kDa mosquito *Aedes aegypti* salivary protein, is an allergen which binds to the IgE of mosquito-allergic subjects. In this Example, an expressed, purified recombinant Aed a 1 (rAed a 1) was characterized to determine if it bound to antibodies directed to the native protein. Additionally, responses to it in mosquito-allergic subjects was investigated to determine its biologic activity, that is activity *in vivo* to elicit an immune response.

Two cDNA segments were ligated together forming the full-length Aed a 1 gene, which was inserted into the baculovirus expression vector pBlueBacHis C. Recombinant baculoviruses were generated by co-infection of Sf9 insect cells with wild-type baculovirus AcMNPV DNA and the recombinant vector. By Western blot using rabbit anti-rAed a 1, the resultant baculovirus were proved to express the 68 kDa rAed a 1 which was secreted into the culture medium as a non-fusion protein. Also, by Western blot the recombinant Aed a 1 showed identical immunological reaction with the native Aed a 1 in the saliva. rAed a 1 in the culture medium was then purified using anion exchange and gel filtration chromatography.

Skin epicutaneous tests with purified rAed a 1 and a commercial crude *Ae. aegypti* extract were performed in 31 subjects with positive reactions in *Ae. aegypti* bite tests and 17 subjects with negative reactions in the bite tests (Figure 5). Immediate wheal and flare were measured 20 minutes after the epicutaneous test, and delayed reactions were measured 24 hours later. Nine of 31 mosquito allergic-subjects (29%) had a positive immediate reaction to rAed a 1, compared to 10 for the commercial extract (31%). In the nine subjects with positive rAed a 1 reactions, the flare sizes induced by rAed a 1 significantly correlated with those induced by mosquito bites ($r = 0.88$, $p < 0.001$) (Figure

6). Also, six of the nine subjects (18%) developed an positive delayed skin reaction to rAed a 1, versus four for the commercial extract (12%). None of the subjects with negative reactions to mosquito bites (0%) showed a positive immediate or delayed reaction to either of the two mosquito preparations.

This Example demonstrates that the recombinant Aed a 1 expressed by the baculovirus system has the same antigenicity and biological activity as the native Aed a 1 present in mosquito saliva and is a major salivary allergen of *Ae. aegypti*.

EXAMPLE 6

IMMUNOLOGICAL CHARACTERIZATION AND CLINICAL USE OF rAed a 2, A 37 kDa RECOMBINANT SALIVARY ALLERGEN OF MOSQUITO *AEDES AEGYPTI*

Mosquito salivary proteins cause allergic reactions in humans. As shown in the Examples herein Aed a 2, a 37 kDa mosquito *Aedes aegypti* salivary protein, is an allergen which binds to the IgE of mosquito-allergic subjects. In this Example, an expressed, purified recombinant Aed a 2 (rAed a 2), was characterized to determine if it bound to antibodies directed to the native protein. Additionally, responses to it in mosquito-allergic subjects was investigated to determine its biologic activity, that is activity *in vivo* to elicit an immune response.

Sf9 insect cells were co-infected with the transfer vector pVL1392/Aed a 2 DNA and wild-type baculovirus. By Western blot using polyclonal rabbit anti-rAed a 2, the recombinant baculovirus was proved to express rAed a 2, which was secreted into the culture medium as a non-fusion protein. The optimal expression of rAed a 2 occurred at 96 hours after infection. rAed a 2 was then purified from the culture medium to homogeneity using anion-exchange (DEAE Sephacel) chromatography. The rAed 2 was able to bind to the IgE of mosquito-allergic sera in Western blot

and ELISA. This binding was inhibited by addition of mosquito head and thorax extract in a dose-dependent manner, showing that the binding of mosquito antigens to human IgE is specific (Figure 7).

Skin (epicutaneous) tests with purified rAed a 2 and a commercial crude *Ae. aegypti* extract were performed in 31 subjects with positive reactions in *Ae. aegypti* bite tests and 17 subjects with negative reactions in the bite tests. Immediate wheals and flares were measured 20 minutes after the testing, and delayed reactions were measured 24 hours later. Three of 31 mosquito allergic-subjects (10%) had a positive immediate reaction to rAed a 2, compared to ten for the commercial extract (31%). In the three subjects with positive rAed a 2 reactions, one subject (3%) developed a delayed skin reaction to rAed a 2, versus four subjects tested with the commercial extract (12%). None of the subjects with negative reactions to mosquito bites (0%) showed a positive immediate or delayed reaction to either of the two mosquito preparations.

Aed a 2 was also shown to be a species-shared allergen, being present in the saliva or salivary gland extracts of 6 *Aedes* and one *Culex* species among the 12 species studied (Figure 8).

We conclude that the recombinant Aed a 2 expressed by the baculovirus system has identical antigenicity and biological activity with native Aed a 2 present in mosquito saliva and that Aed a 2 is a common allergen shared by *Aedes* genus and other species.

EXAMPLE 7

IMMUNOBLOT ANALYSES OF SALIVARY ALLERGENS AND IgE RESPONSES TO THE ALLERGENS IN 10 MOSQUITO SPECIES WITH WORLD-WIDE DISTRIBUTIONS

In this Example, saliva or salivary gland extracts were prepared as described herein above from ten

mosquito species including seven species with world-wide distribution. These species are *Ae. aegypti*, *Ae. vexans*, *Ae. albopictus*, *Ae. togoi*, *Ae. triseriatus*, *Cx. quinquefasciatus*, *Cx. pipiens*, *Cx. tarsalis*, *An. sinensis* and *Cs. inornata*. Proteins from the mosquito preparations were separated by SDS-PAGE and then transferred to nitrocellulose membranes. The membranes were immunoblotted by sequential incubations of the membranes with human serum, monoclonal anti-human IgE, and enzyme-conjugated goat anti-mouse IgG. Salivary allergens were analyzed using a pooled serum from mosquito-allergic subjects.

Individual IgE responses to each allergens were evaluated in 12 mosquito-allergic subjects living in Canada, the USA, and China, as well as in five subjects who were not allergic to mosquito bites. Immunoblotting was also performed using two rabbit antibodies specific to recombinant mosquito salivary proteins, in order to study species-shared allergens.

Three to 16 salivary allergens with molecular masses ranging from 16 to 95 kDa were found in each species (Figure 9). Both species-shared and species-specific allergens were identified by molecular masses, binding to the two rabbit antibodies, and the individual IgE responses to species which were not indigenous to the areas where the subjects lived.

As shown in Figure 10, the existence of species-shared allergens was confirmed by immunoblot using rabbit anti-Aed a 1 and Aed a 2 antibodies, respectively. A 68 kDa allergen which was recognized by rabbit anti-Aed a 1 was found not only in the *Ae. aegypti* extract (Fig. 10A strip #1) but also in the extracts of *Ae. vexans* and *Ae. albopictus* (Fig. 10A strips #2 and #3), but not in the extract of *Cx. quinquefasciatus* (Fig. 10A strip #4). There were no allergen bands found in the PBS control strips (data not shown). Similar results were obtained when rabbit anti-Aed a 2 antibody was used. A 37 to 39 kDa protein

recognized by rabbit anti-Aed a 2 was identified in all 3 *Aedes* species studied (Fig. 10B strips #1 - #3), and in *Cx. quinquefasciatus* as well (Fig. 10B strip #4).

Using the sera from individual subjects who lived in various areas, species-shared and species-specific allergens were identified, because each mosquito species is distributed differently. Allergic sera from Canada (Winnipeg, Manitoba where only *Ae. vexans* is found as a major pest) reacted not only with *Ae. vexans* but also with the allergens of 5 other species which are not found in Manitoba. Similarly, sera from China reacted with the *Ae. vexans* allergens, a species which is not present in China. These data show the existence of species-shared allergens.

Species-specific allergens also existed as evidenced by a 23 kDa *Ae. vexans* allergen which reacted only with the sera from Canada where *Ae. vexans* is abundant.

Salivary allergens elicited higher IgE responses in mosquito-allergic subjects than in non-allergic subjects. Three major *Aedes* species (*Ae. aegypti, Ae. vexans, Ae. albopictus*) had a higher number of allergens which also elicited stronger IgE responses, suggesting that they are major biting species.

During a mosquito bite, the saliva injected may cause a variety of local and systemic adverse reactions, for which young children are at high risk. We report five children age two-four years, evaluated months after "cellulitis" was diagnosed at the site of a mosquito bite because of a large, severe reaction with erythema, edema/induration, and warmth involving an entire body region (periorbital area, hand, foot, or leg), for up to two weeks. Systemic symptoms included fever and irritability. Blood cultures were negative. The diagnosis was confirmed by measuring *Aedes vexans* saliva-specific IgE, IgG, and IgG4 in serum. We also report control children age two-four years with typical reactions to mosquito bites, and mosquito bite-negative

adults.

ELISAs (mean)	Skeeter Syndrome n=5	child controls n=5	adult controls n=10
IgE (U/mL)	1,491	21	13
IgG (U/mL)	277	9	15
IgG4 (U/mL)	7,174	15	14

In Western blotting, sera from children with Skeeter Syndrome reacted with 8-15 *Aedes vexans* salivary antigens. Skin tests with commercial extracts, which contain little mosquito salivary antigens, (Ann Allergy Asthma Immunol 1996; 7:371-6) were not performed.

Throughout this application, various publications, including United States patents, are referenced by author and year and patents by number. Full citations for the publications are listed below. The disclosures of these publications and patents in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

The invention has been described in an illustrative manner, and it is to be understood that the terminology which has been used is intended to be in the nature of words of description rather than of limitation.

Obviously, many modifications and variations of the present invention are possible in light of the above teachings. It is, therefore, to be understood that within the scope of the appended claims, the invention may be practiced otherwise than as specifically described.

Table I. Commercial mosquito extracts and information provided by manufacturers

No.	Name of manufacturer	Lot No	Species	Concentration	Preservative	Use
C1	Hollister-Stier (Miles Canada Inc, Etobicoke, Ont)	D41J3173	*Aedes aegypti*	1:10 w/v	50% glycerin	Epicutaneous test
C2	Hollister-Stier (Miles Canada Inc, Etobicoke, Ont)	H42J5370	*Culex pipiens*	1:10 w/v	50% glycerin	Epicutaneous test
C3	ALK Labs, Inc (Berkeley Biologicals, Berkeley, CA)	635090	*Aedes* species	1:50 w/v	50% glycerin 0.5% phenol	Epicutaneous test
C4	Allergy Labs of Ohio Inc (ALO) (Columbus, OH)	B406212A	*Culex pipiens*	1:20 w/v	0.4% phenol	Epicutaneous test Immunotherapy
C5	Centre Labs (Port Washington, NY)	4G66391	*Culex pipiens*	1:10 w/v 20K PNU/ml	0.4% phenol	Immunotherapy Epicutaneous test
C6	Meridian (Subsidiary of Ventrex Labs, Inc) (Round Rock, TX)	3V1	*Culex pipiens*	1:10 w/v	50% glycerin	Epicutaneous test
C7	Greer Labs, Inc (Lenoir, NC)	B10-33	*Aedes taeniorhyncus*	1:10 w/v 20K PNU/ml	0.4% phenol	Epicutaneous test

Table II. Protein concentrations and epicutaneous test results of commercial and laboratory mosquito extracts

Mosquito extracts (w/v)	Protein concentration (mg/ml)	Skin reactions (mm^2)		Skin reaction/Protein	
		Immediate wheal	Delayed papule	Immediate wheal	Delayed papule
Commercial whole body extracts:					
C1 *Aedes aegypti* (1:10)	3.16	7.5 (2)*	3.0 (0)	2.4	0.9
C2 *Culex pipiens* (1:10)	2.58	14.5 (0.5)	14.0 (0)	5.6	5.4
C3 *Aedes* species (1:50)	1.16	10.5 (0)	5.0 (0)	9.1	4.3
C4 *Culex pipiens* (1:20)	1.24	18.5 (0)	10.0 (0)	14.9	8.1
C5 *Culex pipiens* (1:10)	0.09	0.5 (0)	0.0 (0)	5.6	-
C6 *Culex pipiens* (1:10)	4.85	32.0 (5)	36.0 (0)	6.6	7.4
C7 *Aedes taeniorhyncus* (1:10)	1.67	0.5 (0)	0.0 (0)	0.3	-
Laboratory-made extracts of *Aedes aegypti*:					
Whole body	1.20	11.0 (0)	3.0 (0)	9.2	2.5
Head and thorax	0.62	11.0 (0)	1.0 (0)	17.7	1.6
Salivary gland	0.14	18.0 (0.5)	2.0 (0)	128.6	14.3
Saliva	0.25	19.5 (0)	6.5 (0)	78.0	26.0
Aedes aegypti bite		80 (0)	367 (0)	-	-

* () mean reaction size in the controls

TABLE 3

Skin bite tests and serum mosquito (*Ae. vexans)* salivary gland-specific IgE and IgG levels of the 5 subjects used in this study

Subjects No	Skin bite test (cm^2)		Mosquito specific-IgE (U/ml)	Mosquito specific-IgG (U/ml)
	Immediate wheal	Delayed papule		
1	1.5	11.6	1026	576
2	1.0	9.6	4341	3285
3	2.0	7.2	1067	1054
4	0.0	0	277	140
5	0.1	0	112	80

TABLE 4.

Molecular masses of mosquito antigens in *Ae. vexans*, *Cx. tarsalis* and *Cs. inornata* (kDa).

	Ae. vexans		*Cx. tarsalis*		*Cs. inornata*	
	Binding to		Binding to		Binding to	
	IgE	IgG	IgE	IgG	IgE	IgG
	160	160	160	160	160	160
	110	110	110	110	110	110
	-	-	85	85	85	85
	80	80	-	-	-	-
	-	-	70	70	-	70
	65	65	65	-	65	-
	62	62	62	62	62	62
	-	-	56	56	56	56
	50	50	50	50	50	50
	46	46	46	46	46	46
	43	43	43	43	-	-
	40	40	40	40	40	40
	-	-	37	37	37	37
	32.5	32.5	32.5	32.5	32.5	32.5
	-	-	-	-	30	30
	-	-	-	28	-	28
	-	-	-	25	-	-
	24	24	24	24	24	24
	-	-	22	22	-	-
	18.5	18.5	-	-	-	-
Total number of antigens	12	12	15	16	13	14

TABLE 5

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GA ATT CCG AAA ATG AAA CCC TTG GTT AAA TTA TTC TTG CTA          41
                M   K   P   L   V   K   L   F   L   L

TTC TGT CTG GTA GGC ATT GTG CTT TCC AGG CCC ATG CCC GAA         83
F   C   L   V   G   I   V   L   S   R   P   M   P   E

GAT GAA GAA CCA GTA GCG GAG GGA GGT GAC GAA GAA ACG ACC        125
D   E   E   P   V   A   E   G   G   D   E   E   T   T

GAT GAT GCT GGA GGT GAT GGC GGC GAA GAA GAA AAT GAA GGT        167
D   D   A   G   G   D   G   G   E   E   E   N   E   G

GAA GAG CAT GCT GGA GAT GAG GAT GCT GGC GGT GAA GAT ACT        209
E   E   H   A   G   D   E   D   A   G   G   E   D   T

GGC AAA GAG GAG AAT ACA GGA CAT GAG GAT GCT GGT GAG GAA        251
G   K   E   E   N   T   G   H   E   D   A   G   E   E

GAT GCT GGT GAG GAA GAT GCT GGC GAA GAA GAT GCT GAA AAA        293
D   A   G   E   E   D   A   G   E   E   D   A   E   K

GAG GAA GGA GAA AAG GAA GAC GCC GGA GAT GAT GCC GGA AGT        335
E   E   G   E   K   E   D   A   G   D   D   A   G   S

GAT GAT GGG GAA GAG GAT AGT ACA GGA GGT GAC GAA GGA GAA        377
D   D   G   E   E   D   S   T   G   G   D   E   G   E

GCT AAC GCT GAA GAC AGT AAA GGT AGT GAA AAG AAC GAT CCG        419
A   N   A   E   D   S   K   G   S   E   K   N   D   P

GCC GAT ACA TAT AGA CAG GTG GTT GCA TTA CTC GAC AAG GAT        461
A   D   T   Y   R   Q   V   V   A   L   L   D   K   D

ACC AAG GTG GAT CAC ATC CAG AGT GAG TAC CTT CGA TCA GCA        503
T   K   V   D   H   I   Q   S   E   Y   L   R   S   A

CTG AAC AAC GAT TTA CAA TCA GAA GTG AGA GTT CCG GTG GTG        545
L   N   N   D   L   Q   S   E   V   R   V   P   V   V

GAA GCT ATC GGG AGG ATT GGA GAC TAT TCC AAG ATT CAA GGA        587
E   A   I   G   R   I   G   D   Y   S   K   I   Q   G

TGC TTC AAA TCG ATG GGT AAA GAT GTA AAG AAA GTT ATC AGC        629
C   F   K   S   M   G   K   D   V   K   K   V   I   S

GAA GAG GAG AAG AAA TTT AAG AGC TGC ATG AGT AAG AAG AAA        671
E   E   E   K   K   F   K   S   C   M   S   K   K   K

AGC GAG TAT CAG TGC TCG GAG GAC AGT TTT GCG GCT GCC AAG        713
S   E   Y   Q   C   S   E   D   S   F   A   A   A   K

AGC AAA CTT TCG CCA ATA ACC TCT AAG ATT AAA TCC TGT GTT        755
S   K   L   S   P   I   T   S   K   I   K   S   C   V

TCA TCC AAA GGA CGT TAA TGT TAT CAT AGT AAG CCA TGA ATT        797
S   S   K   G   R   Z

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AAA AAA AAA AAA AAA AAG GAA TTC                               863
```

TABLE 5 (continued)

Primary nucleotide sequence of the Aed a 3 cDNA and its deduced amino acid sequence. The clone was sequenced several times in both directions by the Sanger (1977) method using a US Biochemicals sequence kit. The putative secretory signal peptide is underlined. A translation initiation codon (ATG) and consensus polyadenylation signal sequence (AATAAA) are shown in bold. The sequence data have been deposited in the GenBank databases under accession No. AF001927.

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CLAIMS

What is claimed is:

1. A mosquito extract consisting essentially of antigens related solely to allergens in mosquito saliva.

2. The mosquito extract according to claim 1, wherein said antigens are selected from the group consisting essentially of rAed a 1, rAed a 2, and rAed a 3.

3. The mosquito extract according to claim 1, including twelve antigens from the saliva of *Aedes vexans*.

4. The mosquito extract according to claim 1, including eight antigens from the saliva of *Aedes aegypti*.

5. An isolated and purified recombinant mosquito salivary antigens for use in skin test, immunoassays and immunotherapy for allergic reactions to mosquito bites.

6. The isolated and purified recombinant mosquito salivary antigens according to claim 5 wherein said allergen is produced from a cDNA including an IgE-binding protein or fragment thereof or analogue thereof found in mosquito saliva.

7. The isolated and purified recombinant mosquito salivary antigens according to claim 6 wherein said allergen is selected from the group including rAed a 1, rAed a 2, and rAed a 3.

8. The mosquito extract according to claim 1 wherein said allergen shares common allergenicity with at least two species of mosquitoes.

9. The mosquito extract according to claim 8 wherein said species are selected from the group including *Aedes vexans* and *Aedes aegypti*.

10. The mosquito extract according to claim 1 wherein said allergens are species specific.

11. A method of skin testing and determining the undertaking of immunotherapy by

administering isolated and purified mosquito salivary allergens to a patient; and

recognizing a skin reaction thereto as a positive indication of needing therapy.

12. The method of skin testing according to claim 11 including the steps of selecting the allergens to share common allegenicity among the mosquito species common to a geographic area for which testing and treatment is required.

13. The method of skin testing according to claim 11 including the steps of selecting the allergens to be species specific for each mosquito common to a geographic area for which testing is required.

14. The method of skin testing according to claim 11 including the step of selecting a combination of allergens with common specifications among species and specificities that are species specific to effectively represent the mosquito distribution of a geographic area for which testing and immunotherapy is needed.

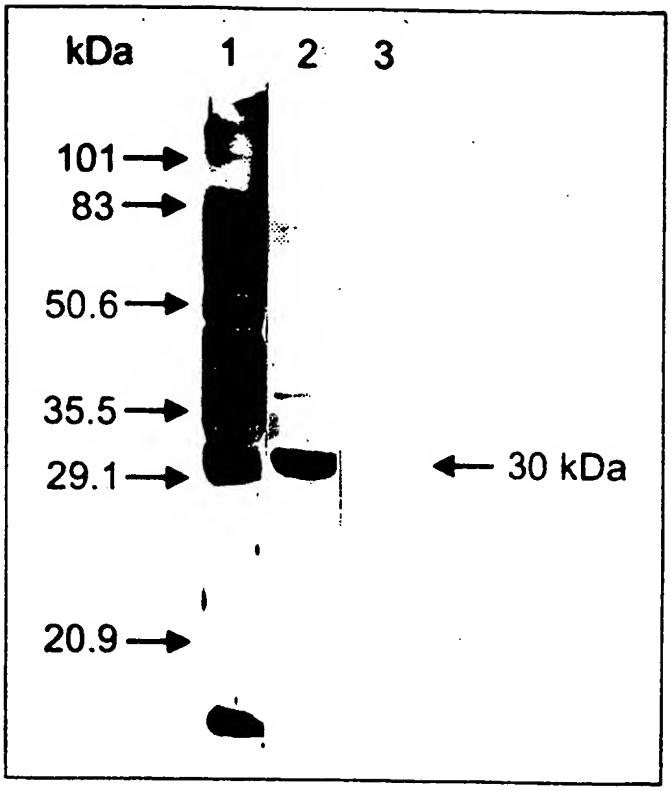
15. A list for performing the method of claim 11.

16. An immunoassay for measurement of mosquito salivary allergen-specific IgE and IgG, said assay including isolated and purified recombinant mosquito allergen as a substrate to which allergen specific IgE and IgG binds.

17. Antibodies directed against recombinant salivary allergens of mosquitoes, said allergens produced by a cDNA encoding or IgE binding protein or fragment thereof or analogue thereof found in mosquito saliva.

18. An antibody of claim 17 selected from the group including recombinant rAed a 1, rAed a 2, or rAed a 3.

19. An antibody of claim 17 wherein said antibody is polyclonal or monoclonal.



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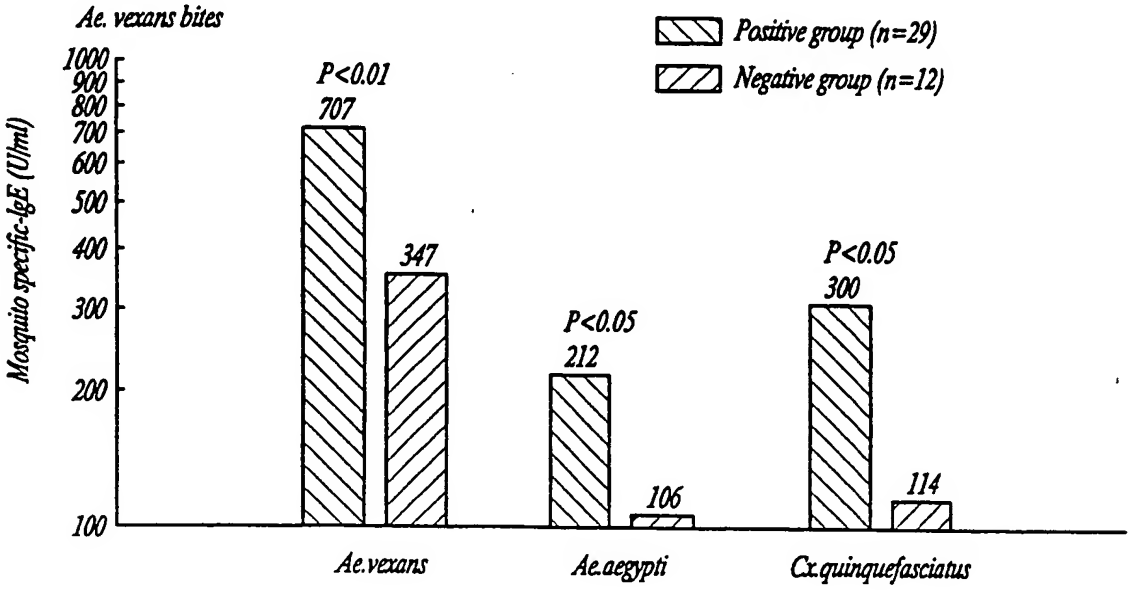


Fig-2A

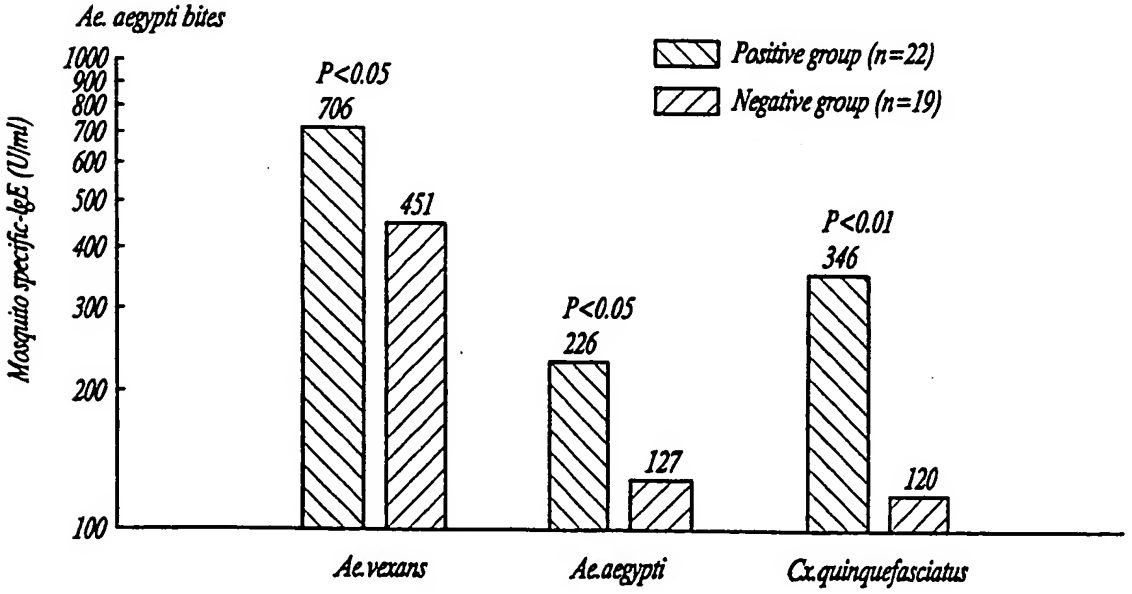


Fig-2B

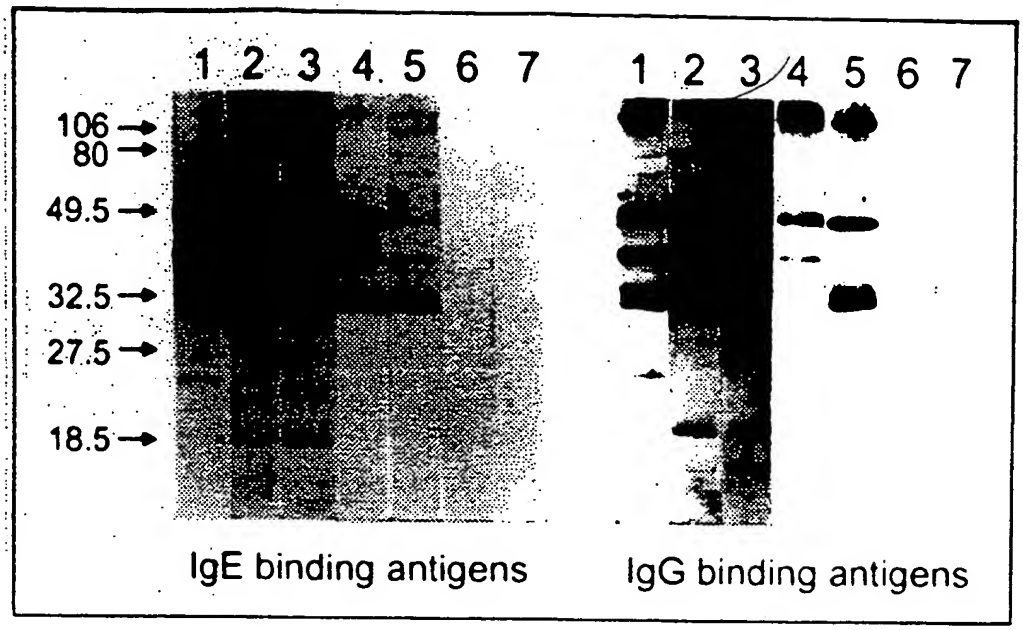


Fig-3

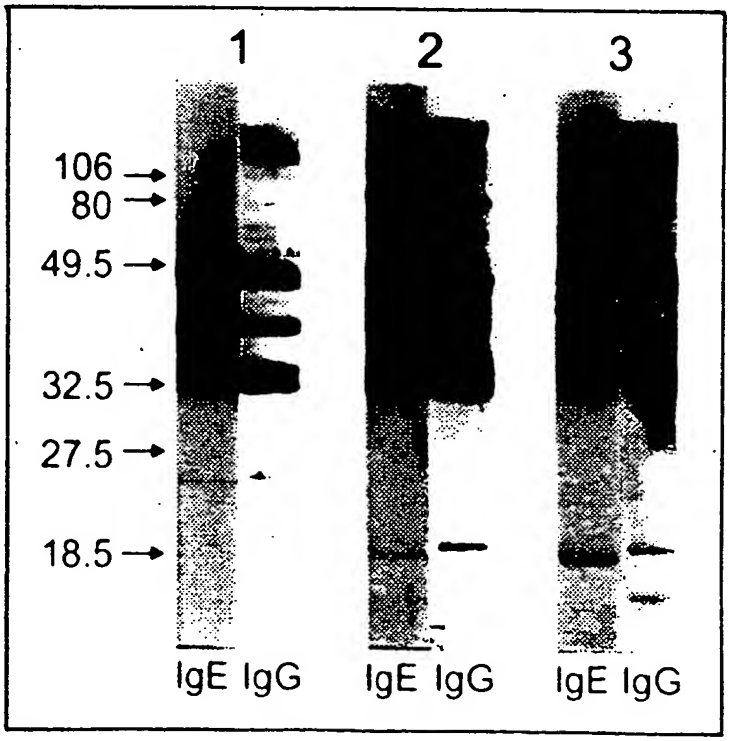
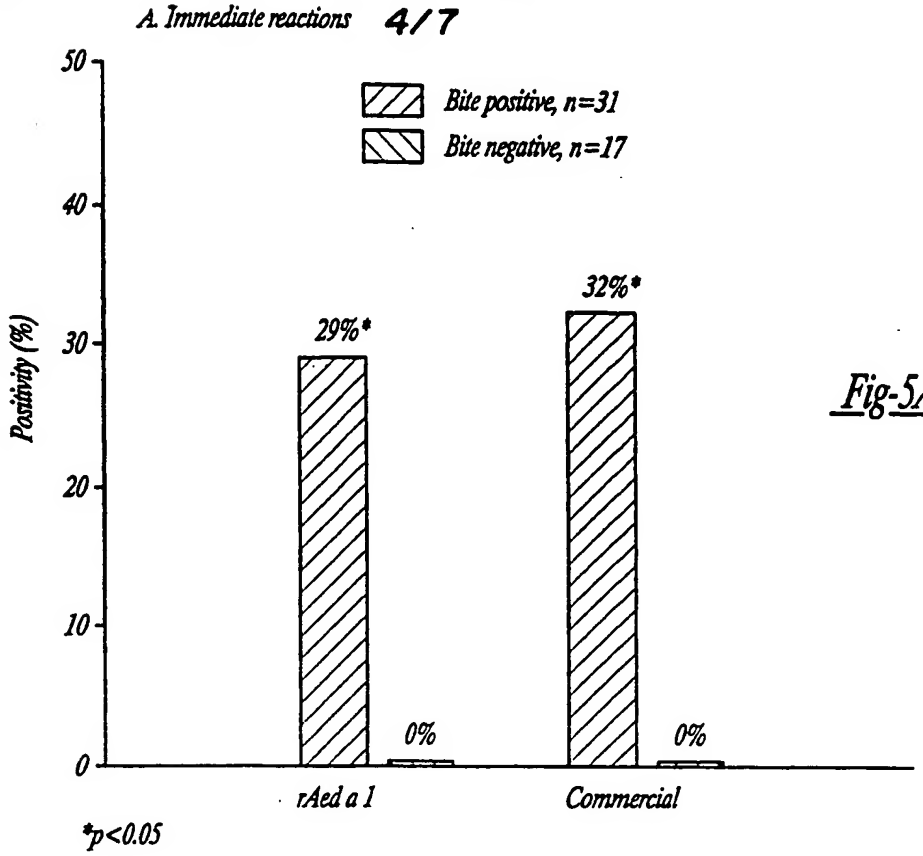


Fig-4



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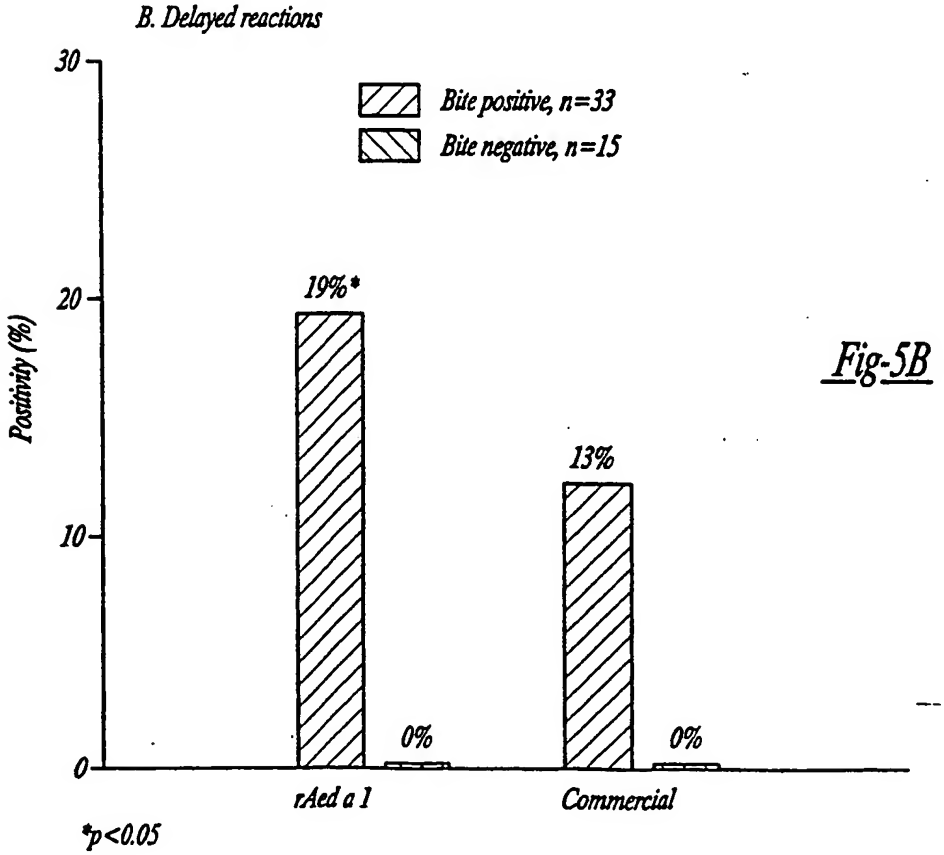

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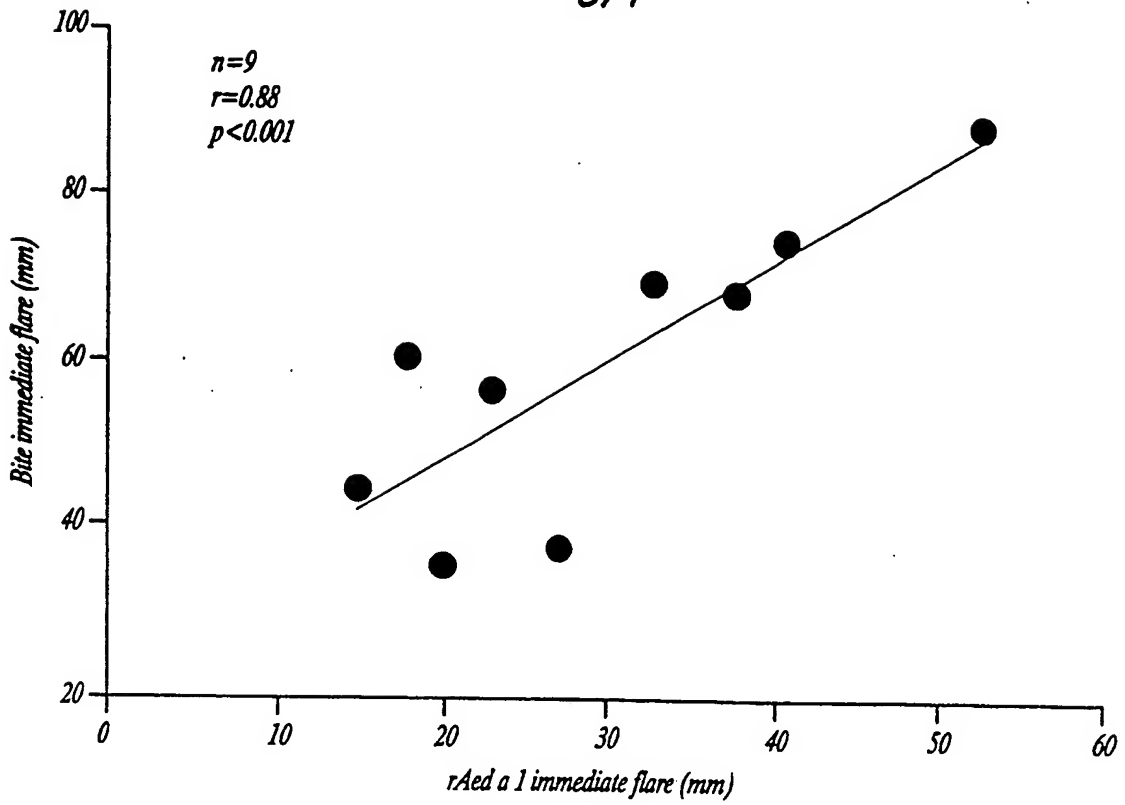


Fig-6

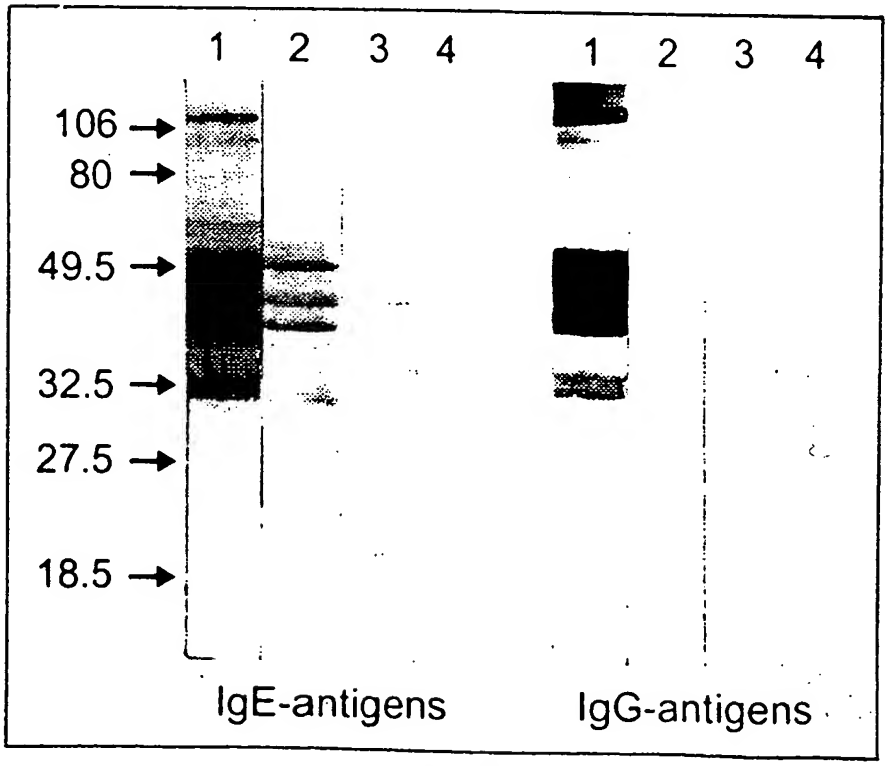
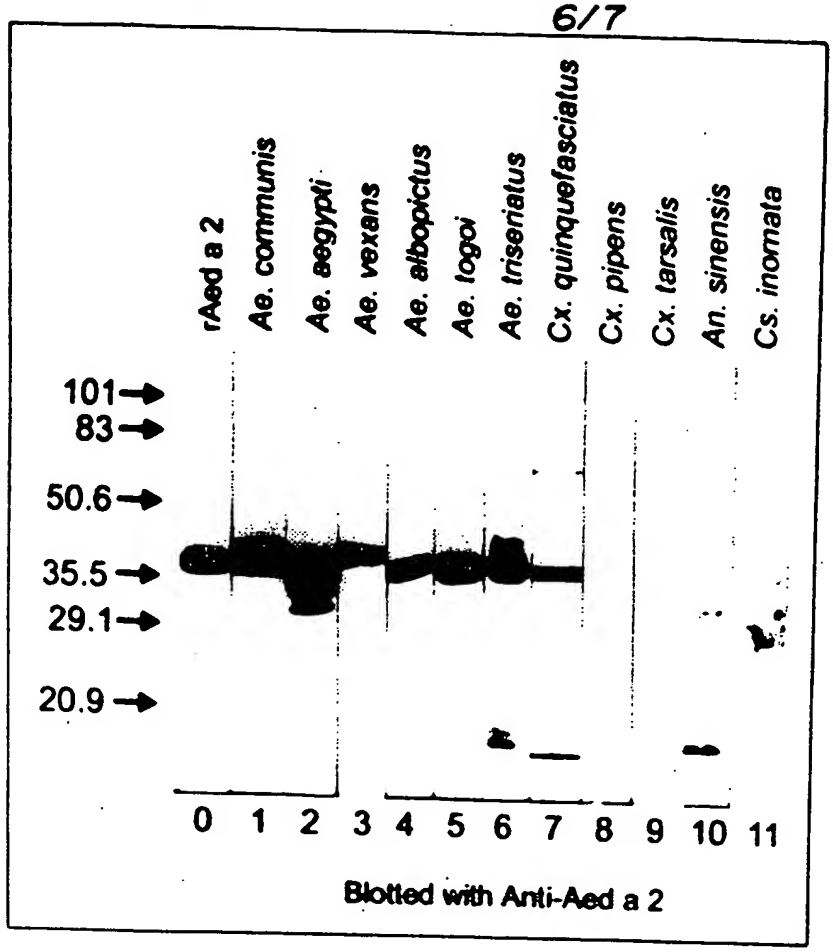


Fig-7


rAed a 2
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Cx. pipens
Cx. tarsalis
An. sinensis
Cs. inornata
101
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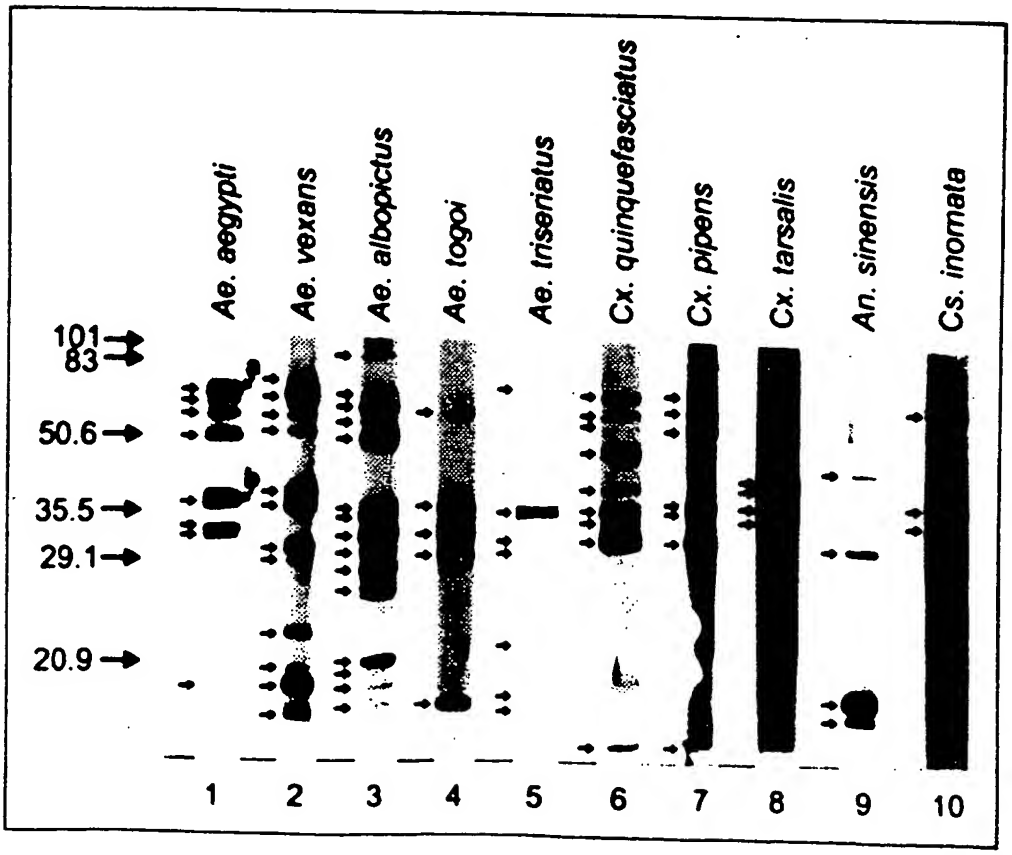
50.6
35.5
29.1
20.9
0 1 2 3 4 5 6 7 8 9 10 11
Blotted with Anti-Aed a 2

Fig-8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20.9
1 2 3 4 5 6 7 8 9 10

Fig-9

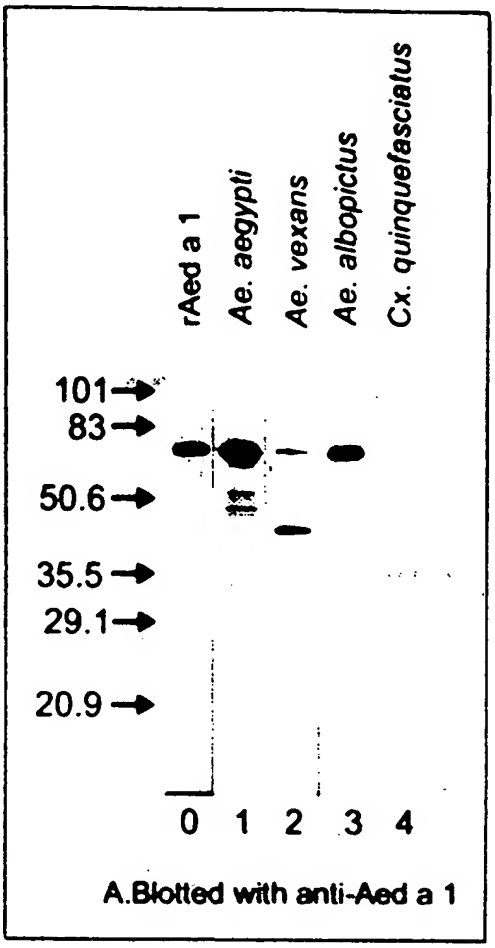

Fig-10A

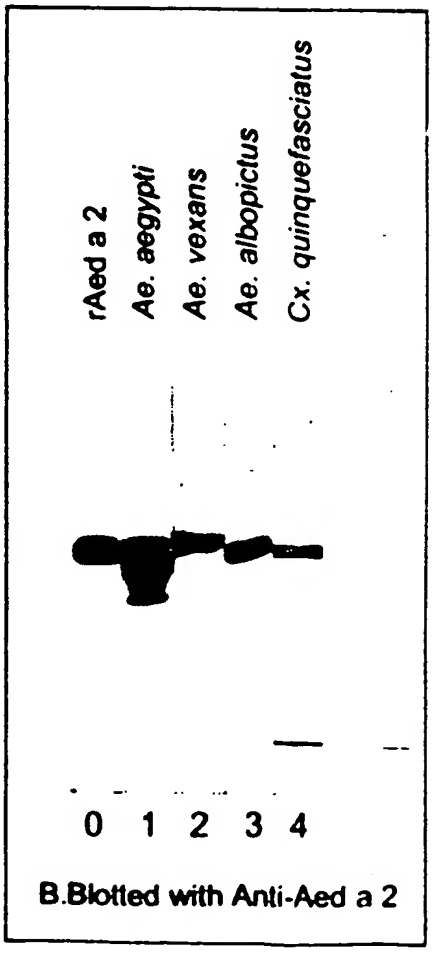

Fig-10B

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Peng, Zhikang
Simons, F. Estelle R.
Kohn, Kenneth I.

(ii) TITLE OF INVENTION: RECOMBINANT MOSQUITO SALIVARY ALLERGENS

(iii) NUMBER OF SEQUENCES: 3

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Kohn & Associates
(B) STREET: 30500 Northwestern Hwy. Suite 410
(C) CITY: Farmington Hills
(D) STATE: Michigan
(E) COUNTRY: US
(F) ZIP: 48334

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Montgomery, Ilene N.
(B) REGISTRATION NUMBER: 38,972
(C) REFERENCE/DOCKET NUMBER: 2595.00027

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 248-539-5050
(B) TELEFAX: 248-539-5055

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 863 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Aedes aegypti

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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INTERNATIONAL SEARCH REPORT

Internal Application No
PCT/IB 98/01961

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/12 C07K14/435 G01N33/68 C07K16/18 A61K38/17

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K C12N G01N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PENG Z. ET AL: "Cross-reactivity of skin and serum specific IgE responses and allergen analysis for three mosquito species with worldwide distribution." JOURNAL OF ALLERGY AND CLINICAL IMMUNOLOGY, (1997) 100/2 (192-198). REFS: 36 ISSN: 0091-6749 CODEN: JACIBY, XP002098985 see the whole document	1-19

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
7 April 1999	20/04/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Oderwald, H

3

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
O,X	YANG J ET AL.: "Production and identification of monoclonal antibodies to mosquito 'Aedes (Ae.) aegypti! salivary allergens" JOURNAL OF ALLERGY AND CLINICAL IMMUNOLOGY, (1997) VOL. 99, NO. 1 PART 2, PP. S152. MEETING INFO.: JOINT MEETING OF THE AMERICAN ACADEMY OF ALLERGY, ASTHMA AND IMMUNOLOGY, THE AMERICAN ASSOCIATION OF IMMUNOLOGISTS AND THE CLINICAL IMMUNOLOGY SOCIETY , XP002098986 see abstract number 613 see the whole document ---	1,2,5-9, 16-19
O,X	XU, W. ET AL: "Mosquito allergy: Expression, purification, and characterization of Aed a 2, an Aedes (Ae.) aegypti salivary allergen." JOURNAL OF ALLERGY AND CLINICAL IMMUNOLOGY, (1997) VOL. 99, NO. 1 PART 2, PP. S152. MEETING INFO.: JOINT MEETING OF THE AMERICAN ACADEMY OF ALLERGY, ASTHMA AND IMMUNOLOGY, THE AMERICAN ASSOCIATION OF IMMUNOLOGISTS AND THE CLINICAL IMMUNOLOGY SOCIETY , XP002098987 see abstract number 614 see the whole document ---	1,2,5-9, 11,15-19
X	XU W ET AL.: "Molecular cloning and characterization of the gene encoding a 30 kDa salivary allergen of mosquito Aedes aegypti (accession number AF001927)." EMBL SEQUENCE DATABASE,26 May 1997, XP002098988 Heidelberg, Germany see the whole document ---	1,2,5-9
P,X	WO 98 04274 A (UNIV MANITOBA ;PENG ZHIKANG (CA); SIMONS F ESTELLE R (CA); KOHN KE) 5 February 1998 see the whole document -----	1-19

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IB 98/01961

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 5-7 and 11-14 are directed to a method of treatment or to a diagnostic method of the human/animal body (as far as in vivo methods are concerned), the search has been carried out and based on the alleged effects of the compound/composition.

2. [X] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
Claim 15: the search has been restricted to the claimed mosquito allergens.

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/IB 98/01961

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9804274 A	05-02-1998	AU 4049597 A	20-02-1998